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)ME RELATIONS OF THE CROWNGALL ORGANISM TO ITS HOST TISSUE [1]

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INTRODUCTION

For a number of years crowngall has been reported to be a limiting ctor in Wisconsin in the cultivation of certain cane fruits. A recent rvey by Jones (2)[3] revealed its presence in practically all the com- rcial cane fruit plantings in the State, and confirmed the earlier dgments as to its economic importance. Consequently, the writer dertook an investigation of this disease in the summer of 1920, with e aim of directing his efforts primarily along lines which promised to y the foundation for more effective control measures.

In the early stages of this work it was decided to conduct preliminary udies upon the tomato, since it can be grown easily in the greenhouse winter and is very susceptible to crowngall. While tomato stems ere being inoculated by punctures, it was noticed that a water-soaked ea promptly appeared about the point of entry of the needle.

It seemed evident that this darkened area, which tended to be parallel the long axis of the plant, was caused by the occupancy of the inter- llular spaces by liquid (Pl. 1, F, G). Sometimes, as seen from the rface, this water-soaked area attained a length of more than a centi- eter, being especially conspicuous if the plant was quite turgid. No rticular significance was attached to this observation until it was ted that the subsequently developed tumor appeared to conform sely in outline with this water-soaked tissue. Inoculations were then ade in which the outlines of the water-soaked regions were marked ith India ink. When the galls developed, they were found to coincide most exactly in outline with the marked areas (Pl. 1, G, H). These sult- did not seem to accord with the generally accepted idea that e gall developed as a result of the stimulus from organisms which had ined entrance to the interior of the cells (*p. 5, p. 2; 9, p. 287; 10, . 470*). The observed facts seemed rather to indicate the possibility that e organism began its activities in the liquid which occupied the inter- llular spaces around the puncture.

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[2] The writer takes pleasure in expressing his indebtedness to Dr. L. R. Jones and Dr. G. W. Keitt, of the University of Wisconsin, and also to other members of the faculty, especially Dr. S. Eckerson and Dr. E. J. Delwiche, for many valuable suggestions in the prosecution of these studies.
[3] Reference is made by number (italic) to "Literature cited," p. 132.

Since further preliminary experiments confirmed these earlier obser tions, it was decided to extend the scope of the investigation to inclu a study of some of the relations of *Bacterium tumefaciens* Smith a Town., to its host tissues before proceeding with applied aspects of t problem. A report on this work follows.

The crowngall organism for these studies was secured at Madis Wis., in 1920, from a gall found on a Kansas raspberry. Isolations we made by the poured plate method from portions of the gall which h been sterilized on the surface with mercuric chlorid. After the paras was isolated, its pathogenicity was proved on both tomato and raspber Three successive platings and reisolations from the original culture the organism made the author reasonably confident that he was worki with a single strain. Repeated inoculation experiments have shown to be actively pathogenic as evidenced by the production of characte istic galls. Cultural studies (in which the author is indebted to L. K. Jones for cooperation) have shown that it corresponds with Smit description of the crowngall organism (*12, p. 105-127*), as isolated fr the daisy, in all but a few respects.

The size limits of the raspberry organism on agar cultures were fou to be 1 to 1.6 × 0.4 to 0.7μ as compared with 1 to 3 × 0.4 to 1.8μ for t daisy organism (*12, p. 106*). The size of the majority was found to 1.1 × 0.5μ compared with 1.2 to 2.5 × 0.5 to 0.8μ for the daisy organis The raspberry organism turned litmus milk pink slowly, and remains viable on culture media as long as six months, while the daisy organis never turned the litmus milk pink and lived only four or five weeks culture media. These differences are not considered to be of practic importance. Smith (*12, p. 127-132*) has noted variations as great a these between different strains of crowngall bacteria. The writer, ther fore, considers that he is dealing with the crowngall organism, *Bacteriu tumefaciens*.

MODE OF ENTRY OF THE ORGANISM INTO THE HOST TISSUES

Many writers on crowngall have stated that wounds are necessa for the initiation of infection. In order to determine whether or n the organism could enter without the intervention of wounds, the follow ing experiment was performed.

On the stem of each of 2 dozen tomato plants 10 droplike mass from a pure dextrose agar culture of the raspberry strain were applie with a camel's-hair brush. Six more plants were treated in a simil manner, but in addition a puncture was made into the stem throu the center of each mass. After four weeks not a single gall had develope from the 240 masses where there were no wounds, whereas 100 per cen infection had appeared in the case of the parallel inoculations accom panied by punctures.

This experiment was supplemented by one in which tomato seed which had been sterilized on the surface with 1 to 1,000 mercuric chlor solution were planted in three 2 by 10 inch glass tubes partly fille with sterilized soil and in two containing synthetic agar. When th plants had grown sufficiently, the crowngall organism was introduce to both the agar and soil in large quantities in a way that precluded the possibility of wounding the experimental plants in the proces At the same time, two of the plants were inoculated near the apex of th stem to test their susceptibility. The inoculations were made

December 15, 1920. On February 21, 1921, those that had received the inoculation at the top showed well-developed tumors. No other trace of gall could be found on any of the plants. Pure cultures of the organism were reisolated from both the soil and the agar. These readily infected other tomato plants when introduced into them. It appears, therefore, that infection did not occur in the tomatoes from organisms in the soil or agar in the absence of wounds.

Ten inoculations were made with a hypodermic syringe into the hollow pith cavities of castor bean petioles and stems. Varying amounts of a suspension of the organism ranging from 0.2 cc. to 1 cc. were injected. On October 4, 1922, three months later, these inoculations were examined. Well-developed galls were found at the punctures, but nowhere else, even though in some cases the path of the inoculum as it flowed down the pith cavity was traceable by a more or less dark stain. Plates were poured from this discolored region. The organism appeared in abundance and its identity was subsequently established by infection of tomatoes. Repetition of these experiments gave similar results. This work simply confirms the conclusions of earlier writers, namely, that under ordinary circumstances wounds are necessary for crown-gall infection.

Since wounds appear to be necessary for infection, it seemed desirable to inquire into the relation of size or character of the wound to gall development. This seemed especially pertinent in its possible relation to the associated water-soaking of the tissues. Glass and quartz rods were drawn out to make needles of different sizes ranging from 30μ to 385μ in diameter. A series of punctures were made with these needles through masses of the gall organism into tomato stems. After 25 days galls had developed, the diameters of which bore a more or less direct relationship to the diameters of the punctures (Pl. 1, A to E). Since the larger punctures released more liquid into the intercellular spaces, it appears that the size of the gall may be roughly proportional to the volume of tissue in which the intercellular spaces are flooded with liquid. A repetition of this experiment gave similar results.

The experiments outlined above and others of like import raised the question whether the inoculum must be inserted into the injured tissue or if the organisms might enter from the surface through the wounds. Consequently, 50 inoculations were made by puncturing through masses of bacteria which had been applied to tomato stems with a camel's-hair brush. In a parallel series, 50 punctures were first made with a sterile needle, and the organism was applied with a brush a few minutes later. Another series in which 50 sterile punctures were made in a similar manner, but without the application of any bacteria, served as controls. In this experiment not more than 10 punctures were made on a single plant. After five weeks galls had developed at every puncture where the organisms were applied while none had appeared on the controls. The developing galls showed no difference in relation to the two methods of inoculation.

The forces which govern this entry of the organism into the tissue are not definitely understood. The bacteria might conceivably be influenced by any or all of such factors as the collapse of drying tissue, negative pressure, sap rise, and motility with or without a chemotactic stimulus.

To test the possible relation of chemotaxis, very small capillary glass tubes were filled with expressed tomato stem sap. These were placed

in a hanging-drop preparation of a suspension of actively motile crown-gall organisms in sterile distilled water in a manner similar to that described by Pfeffer (*4*). In a few minutes the bacteria were observed collecting in the tube. At the end of a half hour the motile organisms were thickly concentrated in the tube, while no active ones were observed outside. This experiment was repeated eight times with tubes of different diameters. In addition, it was tried with the positions of the bacterial suspension and the tomato sap reversed. In every case the motile bacteria collected in the liquid expressed from the tomato stem. It appears therefore, that this tomato sap exerts a positively chemotactic stimulus upon the crown-gall organism, which alone may be sufficient to account for the entrance of the bacteria into a puncture.

How long one of these individual punctures might serve as an infection court seemed an important point. One hundred and fifty punctures were made into tomato stems, and at intervals of time bacteria were applied to the punctures with a camel's-hair brush. The plants remained in an open greenhouse throughout the experiment. The results, recorded at the end of three weeks, appear in Table I.

TABLE I.—*A summary of results of experiments on the length of time that a puncture may serve as an infection court for B. tumefaciens*

Number of punctures.	Time between puncture and application of organism.	Infection after three weeks' incubation.	
		Number.	*Per cent.*
10	5 minutes	10	100
10	2 hours	10	100
10	4 hours	10	100
20	1 day	19	95
20	2 days	12	60
10	2½ days	9	90
10	3 days	7	70
10	4 days	2	20
10	5 days	0	0

Under more favorable conditions, when the plants were placed in a moist chamber for 24 hours following the application of the bacteria, all infections were secured from 10 punctures that were six days old and from 2 infections from 10 punctures that were seven days old. Eight and nine-day-old punctures produced no galls. A repetition of this experiment gave similar results.

Although it is evident that conditions are important in determining the length of time a puncture may serve as an infection court, these experiments show that infection is very readily accomplished within the first day, and that it may under favoring conditions take place when the application of the organisms is delayed until as long as seven days after wounding the tissue. These results appear to be incompatible with the conception (*6, p. 170*) that the bacteria first establish themselves in wounded cells. It seems unlikely that in the case of such delayed infection any cell walls which might have been punctured at the time of the original wound would still offer open channels for the penetration of the bacteria into the living cells. Before seven days had elapsed the injured cells probably would either have died or healed the ruptures, provided they were capable of the latter.

For the purpose of determining whether punctured cells were necessary for the development of a tumor, the following experiment was devised. Thirty inoculations were made into tomato stems in the usual manner. After 24 hours a red hot needle was passed through the stem in as nearly as possible the position occupied by the inoculating needle. This treatment killed not only the cells that had previously been injured but also all those around the puncture for approximately 1 mm. in every direction. Sixty more inoculations were treated in the same manner, except that the burns were made at the end of an hour. In each case 30 controls were burned. After two weeks 100 per cent infection was found developing above and below the burned inoculations, while the controls showed no signs of proliferation (Pl. 1, I to K). This experiment was repeated on 40 more punctures, with the variation that the bacteria were applied with a camel's-hair brush to the surface of the burn. Here also 100 per cent infection was secured above and below the burn. It seems unlikely that any cells which had been able to survive the heat could have had injuries in the walls through which the bacteria might have entered. These experiments indicate, therefore, that the bacteria exert their influence from some position outside the cells. This position, to begin with, at least, is probably in the liquid which penetrated the intercellular spaces following the puncture.

MIGRATION OF THE ORGANISM

In order to understand more clearly the relations of the organism to the liquid in the intercellular spaces, it seemed advisable to make observations on its motility both in water and in expressed plant juice.

For studies in water, bacteria from a two-day agar culture were mounted in sterile distilled water in a hanging-drop preparation. Observations were made on the length of time it took motile bacteria to move across the field of the microscope. No correction was made for the deviation of the bacteria from a straight line. The average of a dozen measurements showed them to move at approximately the rate of 1 mm. in one minute. Repetition of these measurements gave confirmatory results.

Similar tests were made in which expressed tomato sap was substituted for sterile distilled water. The motility of the organism did not appear to be materially changed so long as the preparations were fresh. However, the organisms in the tomato sap retained their motility longer than those in the sterile distilled water.

This gives an easy explanation of how the organisms might reach the limits of the region flooded with the liquid which was released by the puncture. Further studies of the range of migration were made in relation to extensive wounds caused both by mechanical crushing and by freezing.

Large wounded areas were produced on the stems and petioles of seven tomato plants by pressure from a glass rod. These bruised areas were made on one side of the stems and extended for longitudinal distances varying from 4½ to 10 cm. Crowngall bacteria were then introduced by a needle puncture into the lower portion of the injured region of five of the plants, while two were retained as controls. After four weeks, continuous well developed galls had extended for several centimeters above the puncture that had received the bacteria (Pl. 2, A, B). In no

case did the galls extend materially into the unbruised tissue. No proliferations were noted in the controls. In one case, a more or less continuous gall developed over the whole length of the crushed tissue. In the other four, the tumors extended over only about two-thirds or three-fourths of the length of the wounded area. In these cases it seemed possible that the bacteria failed to reach the limit of the crushed region because their path was obstructed by some break in the continuity of the liquid. A repetition of this experiment gave similar results.

Confirmatory evidence was obtained when the wound was produced by freezing. For this purpose, a carbon dioxid tank was provided with a single jet, from which the gas was projected against the tissue. By this means each of six plants was treated so that one side of the stem was frozen for a longitudinal distance of from 4 to 6 cm., with the result that a water-soaked area extended over this distance. Bacteria were then applied through a puncture at the lower end of the water-soaked area. After 18 days galls had developed at intervals all along the frozen areas (Pl. 2, C), but the more prominent proliferations were produced at the points of puncture. Controls showed no such proliferation. No evidence of any tumor strands could be found between the galls at the places of puncture and the other tumors by a study of free-hand sections.

These experiments indicated that the bacteria could travel several centimeters at least if they were given a continuous channel of fluid through which they might pass. This raised the question of whether or not they might pass through the tracheae.

Consequently, a tomato stem was heavily inoculated in one place in such a manner that the punctures passed through in several directions so that the penetration of some of the tracheae appeared certain. Then with a sterile needle, punctures were made through the stem at intervals from the apex down to the point of inoculation with the expectation of penetrating some of the same vessels that were injured at the point of inoculation. The plant wilted as the result of such treatment, but recovered after a day. In three weeks well-developed galls were found at the point of inoculation, and smaller ones were observed at irregular intervals in two-fifths of the sterile-needle punctures (Pl. 2, H, I). The farthest one was at a distance of 7 cm. from the point of inoculation. These results are interpreted to indicate that those punctures about which no galls were produced had not penetrated vessels which contained bacteria. The same method was tried with six other plants. Two of these gave results similar to those just described, but the other four developed proliferations only at the points of inoculation. Control plants which were punctured but not inoculated produced no gall formation.

A further study of the passage of the organism through stem tissue was begun in the laboratory by placing a tomato stem which had been cut under water in a suspension of a pure culture of the bacteria (Pl. 2, D). After an hour and a quarter the stem was cut at intervals of 5, 3, 2, and 1 cm. above the suspension. Sap was pressed out from the cut base of each section that was removed and transferred to agar, where the gall organism developed in abundance. Smears of the sap from 5 cm. above the suspension showed large numbers of the organism. This experiment was repeated five times on tomato and once on raspberry with time intervals varying from 30 minutes to 3 hours and with variations up to 9 cm. in the distance above the point of entry of the organisms. In every case the organism was easily recovered.

In order to determine more clearly whether the bacteria had passed through the tracheae, a tomato stem was tied to a supporting rod and en a section several centimeters long was frozen by means of carbon oxid. On the next day the soft tissue had collapsed and dried, leaving pparently only strands of dead conductive vessels with the firm covering shrunken tissue about them. This treatment did not cause severe lting of the upper part of the plant, especially when part of the top as pruned off. The tissue which had been frozen was allowed to dry an dditional day to further guard against the occurrence of a moist passage r the bacteria outside of the vascular tissue. Then the stem was cut f and placed in a suspension of the bacteria (Pl. 2, E), as previously escribed. Two hours later the bacteria were recovered in large numers from the interior of the stem above the collapsed area, but not in uch great abundance as in the earlier experiments. This may have been ue to a reduction in the flow of sap through the bundles.

These experiments were modified so that the stem could be cut part ay through under a suspension of the gall organism. For this purpose cylinder of cork was divided in two and fastened around the stem with ins. Upon this as a base was placed a short piece of large rubber tubing hich had been cut down the side for convenience in locating it around he stem. The whole was sealed with vaseline so that it formed a cup bout the stem (Pl. 2, F). This was filled with a suspension of the organism and a transverse incision was made in the stem below the surface f the fluid. The suspension and cup were removed at the end of 5 hours. After 48 hours punctures were made in the stem with the tip of a sterile nife at intervals from the top down to the cut. Eight weeks later four galls had developed out of eight punctures. One of these was 8 cm. above he inoculation cut. Four repetitions of this experiment gave like results. They produced, in order, three galls from 6 punctures, eight from 14, six from 11, and seven from 9.

While one may not be fully confident of the exact course followed by the bacteria, these experiments suggest that, under favorable conditions, the crowngall bacteria can pass through the tracheae and induce galls at some distance from the point of entry, if they are provided with an avenue of escape from the vessels and suitable circumstances for development. However, there is no evidence to show that they can produce galls if they remain inside these vascular elements.

To determine whether or not the organism occurs commonly in some of the vascular tissue, 60 sterile punctures were made in the stems of tomatoes above three-weeks-old galls. Four weeks later not a single one showed any evidence of tumor formation. So it may be assumed that this parasite may travel in some parts of the vascular bundles if conditions are favorable, but that probably it does not ordinarily induce infection from this position.

EARLIER ATTEMPTS TO LOCATE THE ORGANISM

The experiments so far described have indicated that the crowngall organism begins its relations with its host tissues in the liquid occupying the intercellular spaces about the wounds. The first striking evidence in support of this conception came to the writer's attention in an experiment described on an earlier page. There it was shown that the galls developed rather uniformly throughout the water-soaked area produced by the puncture and closely coincided with it in outline. So from the

beginning it appeared to be the simple and natural suggestion from the evidence that the bacterial invasion not only started in the intercellular spaces but possibly also continued therein in a manner comparable to that of various other bacterial pathogens.

With this question in mind a search was instituted with the hope of detecting the bacteria in situ. In the beginning it was realized that the organism might be located only in certain portions of the gall and might also be more apparent at one time in the stages of development than at another. Consequently, in this search the efforts were not confined to any portion of the stem taken at one time, but were directed to whole sections of stems with galls in different stages of development.

Material was killed in such fixatives as chrom-acetic, Flemming's, Carnoy's, picro-formal, formal-alcohol, and Benda's. Paraffin sections were then cut and stained with carbol-fuchsin, methylene blue, gentian violet, Haidenhain's iron-alum-haematoxylin, safranin, rose Bengal, Giemsa, neutral red, orange G., and light green, either alone or in combination. No satisfactory demonstration of the organism in the tumor tissue was secured. The organisms could not be distinguished, except as Smith states (*11, p. 17*), relatively near the entrance of the needle. However, it was noted that the bacteria could be traced from the puncture out through the intercellular spaces for a short distance (Pl. 3, C), and could also be located in many of the intercellular spaces above or below the puncture. This was quite easy in the cortex and pith where the intercellular spaces were large. Search was of course persistently made for evidence of the occurrence of bacteria within the cells, but they were detected only in those cells which were close enough to the puncture to be subject to injury. In such cases the organisms were present in sufficient quantities to occupy a major portion of the cells (Pl. 3, D). It appears, therefore, that if entrance into the cells is provided for the bacteria, they collect there in considerable numbers. It seems quite improbable, however, that cells showing such abundant invasion should survive.

Efforts were made to locate the bacteria with gold chlorid (*7, p. 15*) and with Gram's stain, employing amyl alcohol as used by Smith (*11, p. 18-19*), but these also gave unsatisfactory results. Such combinations as ruthenium red and methylene blue, used so successfully by Jones (*3, p. 332*) were equally ineffective.

While studying paraffin sections the writer noticed that certain of the walls bounding intercellular spaces took the stain much more deeply than others. This was found to be true of the spaces that had been filled with liquid after the puncture and to which the bacteria had gained access (Pl. 3, A). The bacteria seemed to have exerted an action on the walls which made them stain more deeply. This influence was noticed to extend a short distance into the middle lamella (Pl. 3, B), but its exact range has not been determined. This seemed to be in accordance with the hypothesis that the bacteria were located in the intercellular spaces and possibly to some extent in the middle lamellae. If this were true, a satisfactory explanation would be given alike for the different types of staining shown by different cell walls, and for the development of galls from the region water-soaked by the puncture.

So the problem became one of trying to differentiate between the cell wall, which had been made more sensitive to stain by the action of the bacteria and the organisms themselves. For this purpose sections were

used which showed the bacteria in mass about the puncture. In this case the differentiation was not so difficult because the bacteria were together in large numbers and the walls in these early stages had not undergone any change due to the bacterial action. But this gave no idea of the relation of small numbers of bacteria to the walls that took the stain more deeply.

Since the usual paraffin method had yielded inconclusive results for the writer, as it had earlier for Smith (*8, p. 253*), it seemed evident that some variation in method must be employed if the organism were to be demonstrated in the tissue. At the suggestion of Doctor Eckerson a variety of microchemical tests were performed on free-hand sections of gall tissue in the hope that either the walls or protoplasm of the bacteria would be found to give a characteristic reaction. For the wall the chitosan reaction was tried, but it gave only unsatisfactory results. For protein, the biuret, Berlin blue, and xanthoprotein reactions and Millon's reagent gave no better success, so far as demonstrating the bacteria was concerned.

While these reactions were being tried, observations were made on free-hand sections of tomato galls that varied from one day to three weeks in age. These sections were mounted in water in the usual manner and examined under an oil immersion lens. In the middle lamella, especially of the collenchymal cells, small bacteroid bodies were observed (Pl. 4, A), which, however, varied considerably in size. At the same time, in certain of the intercellular spaces, bacterium-like bodies were observed which were of rather uniform dimensions. These were associated with a yellowing of the adjacent cell walls. They were visible, just as bacteria are in hanging-drop preparation, because of the differences in light refraction. Occasionally, also, as Smith observed (*11, p. 17*), bacteria could be seen in cut cells. However, the fact that they appeared inside the open cells was no proof that they were present in this position before the section was made. If they were in the intercellular spaces, one would expect some to escape as soon as the wall was cut, and then they might appear anywhere on the surface of the section.

This possibility that the bacteria might wash out of the middle lamellae or intercellular spaces into the water during the sectioning made it seem advisable to cut the material dry. The actual practice, with a good sharp razor, proved to be much easier than anticipated. The sections were mounted quickly in a small drop of water, in glycerin, or in lacto-phenol (equal parts of lactic acid, phenol, glycerin, and water). This last medium was the most convenient because it did not dry out like the water, nor did it contain so many annoying air bubbles as the glycerin. At the same time it proved to be an excellent preservative.

The large bacteroid granules observed in the middle lamellae especially between the collenchymal cells were found in observations made in February, 1922, to be associated with the rapidly growing gall tissue but not with the more slowly growing uninoculated tissue (Pl. 4, A, B). However, when an examination was made in June, 1922, while the tomato stems were growing rapidly, similar granules were found in the normal as well as the gall tissue. Their presence seems to be associated with the rate of growth. Polarized light showed them not to be doubly refractive. They lost their identity quickly in 3 per cent hydrochloric acid upon the application of heat, and slowly in the lacto-phenol mounting fluid. They stained both with methylene blue and ruthenium red.

Together with the middle lamellae they were dissolved after treatment with dilute acids followed by dilute alkalies. It appears that they are composed of some pectic substance, possibly calcium pectate.

LOCATION OF THE BACTERIA IN THE INTERCELLULAR SPACES

Evidence has been accumulated which shows that the bacterial granules which were observed in the intercellular spaces bordered by yellowed walls are the crowngall bacteria in situ. These bacterial bodies have been observed to be constantly associated with crowngall tissue in tomato stems through four series of inoculations in which the galls were examined at two-day intervals from the day of inoculation until the galls were 22 days old. At the latter age they showed the characters of maturity—that is, they had well-developed hypertrophic and hyperplastic areas which were abundantly supplied with new vascular elements. The bacterial bodies were found very easily in the early stages and without much difficulty in the later ones because of the yellowing of adjacent walls which accompanies their presence. This yellowing is similar to that which may be observed in connection with various types of injured tissue. Consequently, although crowngall bacteria in the intercellular spaces appear to be accompanied by a yellowing of the surrounding walls, this color does not always indicate the presence of bacteria.

With the aid of polarized light it was observed that the yellowed walls had lost their property of double refraction. It appeared that the bacteria had produced some change in the cellulose of adjacent walls. However, a further consideration of the nature of this action is beyond the scope of this paper.

The phenomena just described were not observed in either sound tissue or tissue that had been punctured but not inoculated. The most likely sources of confusion were found to be the granules of pectic substance already described and deposits of very tiny crystals of calcium oxalate. These latter were observed quite commonly in the gall tissue. Their identity as crystals was very easily established by the use of polarized light.

After the location of the bacteria was ascertained the method of demonstrating them in the paraffin sections became simplified. It appeared that the customary methods had failed because the bacteria produced an effect on the cell wall that made it take up the stain more heavily than did the normal walls. This resulted in a masking of the bacteria when they were not present in very large numbers. It was found also that the intercellular spaces occupied by the bacteria gave a positive protein reaction to Millon's reagent, while the bacteria themselves did not. Whether this substance is produced by the bacteria or by the neighboring cells is not understood, but its presence certainly renders more difficult the demonstration of the bacteria.

It remained to discover a combination of stains that would color the bacteria and still not hide them by staining too deeply the walls and protein substance surrounding them. This was found when dilute carbol-fuchsin was used in combination with light green. Tissue which had been killed in chrom-acetic or formal-alcohol fixatives was dehydrated and embedded in the usual manner. Sections were cut between 6 and 12μ in thickness. The sections were stained for one minute in carbol-fuchsin which had been very greatly diluted (1 part by volume of carbol-fuchsin to 100 parts of water). Then after very rapid treatment with

bsolute alcohol the sections were cleaned and stained by a saturated olution of light green in clove oil, rinsed in xylol, and mounted in alsam.

Slides made in this manner showed the walls acted on by the bacteria nd the xylem walls to be stained red, while the rest of the tissue appeared reen. The bacteria for the most part appeared red along with the ordering tissue (Pl. 3, C, D; 4, C, D; and 5, A D). The crystals of alcium oxalate remained uncolored. The previously mentioned gran- les of pectic substance were never observed in stained preparations.

The bacteria found in these sections are present in larger numbers han was previously supposed. Repeated statements have appeared n the literature that the organisms were scarce (*12, p. 193; 11, p. 18*). hese appeared to be confirmed by the writer's earlier isolations. In iew of the comparisons of microscopic and plate counts made from soil y Conn (*1, p. 10*), it seemed logical to expect a poured plate to show the resence of no more than one-tenth of the number of bacteria that might e distinguished under the microscope. But even with this discount se results from earlier platings showed a comparatively small number f colonies.

The chance examination of a 10-day-old isolation plate revealed an nteresting phenomenon. This plate had been poured from gall tissue which had been treated with mercuric chlorid and washed and crushed before mixing with the agar in a manner similar to that described by Smith (*12, p. 22*). A bacterium-free zone surrounded the portions of tissue for a radius of more than a centimeter, while outside of this region a very large number of colonies had appeared. These were proved by successful inoculation into tomato to be the gall organisms. It seemed quite clear that the treated tissue had an inhibitory influence on the bacteria, due either to the diffusion of the disinfectant used or to some detrimental product of its own.

To determine whether or not a short treatment of the tissue with the mercuric chlorid was inhibiting to the growth of the bacteria, three plates were poured from a suspension of a pure culture of the crowngall organism at a dilution which would produce about 1,000 colonies in each plate. Small pieces of normal tomato tissue which had been dissected out under aseptic conditions and treated with mercuric chlorid were placed in the first and second plates. In the first the tissue was placed 45 seconds in mercuric chlorid 1 to 1,000, as Smith (*12, p. 24*) describes and washed a minute and a half in sterile water, while in the second the treatment with the disinfectant lasted 3 seconds and the washing 10 seconds, as recommended later by Smith (*10, p. 134*). The tissue in the third plate was not treated with the disinfectant, but was placed in sterile distilled water for 30 seconds. After five days the portions of tissue which had been treated with mercuric chlorid for either period had bacterium-free zones around them for a radius of about 1½ cm. Outside of these the bacteria appeared in great abundance. The other fragment, which had been treated only with water, appeared to have exerted no inhibitory effect on the organisms. This experiment was repeated twice with the variation that gall tissue was used as well as normal tissue. The results were confirmatory in every respect.

A few contaminating organisms were secured in the plates with the gall tissue. Some of these were able to tolerate the inhibiting influence.

They apparently diminished the toxic action of the mercuric chlorid sufficiently to permit the crowngall colonies to grow around them. Occasionally, in the clear zones surrounding the treated gall tissue, a contaminator appeared, surrounded by a small zone of colonies of the crowngall organism.

The presence of a large number of causal organisms in crowngall tissue was demonstrated by dissecting out and grinding in white sand and water, under aseptic conditions, a 2 mm. cube of young gall tissue which was produced by inoculation. Dilution plates were poured and the suspension secured. Counts and computations indicated that the block of gall tissue contained approximately 50,000 bacteria capable of producing colonies. Another similar cube was found to contain about 30,000. Under different conditions a piece of gall tissue ¼ c. mm. in volume (1 × 2 × 0.125 mm.) was dissected out under sterile conditions and crushed in a Petri dish. Over 1,100 colonies of the gall organism developed, with practically no contamination. Doubtless many more would have appeared if the plate had not been so crowded. Other similar pieces have produced colonies ranging in number from 18 to 11,600. When these figures are multiplied by 10, one secures numbers consistent with those indicated by the microscopic examinations.

Although the crowngall bacteria have been found in larger numbers than has commonly been supposed, a very wide range of variation has been observed. The number of bacteria secured in culture seems to depend on the number of intercellular pockets of bacteria that are broken open so as to release the organisms. Since the number of these pockets varies with the age of the gall, the portion of the gall selected for isolation, the conditions of growth, etc., considerable differences are to be expected in the numbers of bacteria that appear in plates or sections.

Since it was discovered that mercuric chlorid exerted this inhibiting influence, its use has been abandoned in routine isolations for the crowngall organism. A procedure like the following has been found successful. A 2 to 3 mm. cube of young gall tissue is dissected out under aseptic conditions. This is dropped into 10 cc. of sterile distilled water and crushed. Then dilution plates are poured from the suspension.

A further check on the accuracy of the interpretation of the previously described intercellular bodies as the crowngall bacteria was made by observing their multiplication from free hand sections of gall tissue, subsequently by isolating them from such preparations, and by establishing their identity with the usual cultural and inoculation methods. This was accomplished in the following manner.

Free-hand sections of crowngall tissue were cut under aseptic conditions and mounted in drops of melted nutrient dextrose agar on thin cover slips. After the agar had solidified, each cover slip was fixed with vaseline on a van Tieghem cell as in the preparation of a hanging drop. When the bacteria developed, observations made at about eight-hour intervals showed that the colonies were unusually definitely localized on account of the solid medium. No difficulty was experienced in examining the preparation even with an oil-immersion lens. Colonies were observed to grow consistently from the previously described intercellular bacterial pockets. When the examination was completed, usually when the preparations were two or three days old, the sections in agar were crushed in separate tubes of sterile distilled water, from which dilution plates were poured. These plates have commonly yielded prac-

ically pure cultures of the crowngall bacteria, which were identified by successful inoculations.

From the foregoing observations and experiments we believe it may safely be concluded that the crowngall bacteria are located in certain intercellular spaces of the host tissue, and that they are present in larger numbers than has been supposed.

No attempt has been made in this paper to describe the responses of the host to the bacteria in the intercellular position. These activities, with special emphasis on the formation of the "tumor strands," will be treated at a later time.

SUMMARY

(1) Crowngall infection in tomato stems was found to take place only through wounds.

(2) It was not found to be necessary for infection that the organisms should be carried into the tissue at the time of puncture. Successful results followed their application to the surface of wounded tissues. They were observed to have a positively chemotactic response to expressed tomato sap, which could account for their entrance following surface application. Under favorable conditions infection was induced by organisms applied to the surface as long as seven days after the puncture.

(3) When needle punctures were made into turgid tomato and tobacco stems, the intercellular spaces above and below for several millimeters became occupied by liquid and appeared water-soaked. When puncture inoculations were made, the galls which developed were found to coincide closely in outline with these water-soaked regions.

(4) Galls of different sizes were induced experimentally by varying the sizes of the inoculating needles and consequently the extent of the water-soaked areas.

(5) A half hour after inoculations were made in the usual manner by punctures, a red-hot needle was inserted in the path of the inoculating needle. It is believed that this destroyed all the cells that had previously been ruptured. The subsequent development of galls from the outer margin of the water-soaked region is interpreted as indicating that the bacteria are not dependent upon gaining access to ruptured cells, and is, on the other hand, consistent with the idea that they are distributed in the liquid in the intercellular spaces.

(6) When a continuous channel of liquid was provided in the tomato stem either by mechanical pressure or by freezing, the bacteria migrated and produced galls several centimeters from the point of inoculation. The subsequent gall development occurred over the area approximately corresponding to this original water-soaking of the tissue.

(7) When the organisms were introduced into a wound which cut some of the vascular bundles, they appeared to travel in some part of the conductive tissue, probably the tracheae, and when a wound enabled them to escape, induced proliferation. This type of infection is not considered a common occurrence in nature.

(8) The usual methods of staining failed to demonstrate the bacteria in situ. This appeared to be due to the staining of the adjacent cell walls and of the substance which occurred in the intercellular spaces which was of similar intensity to that of the bacteria, thus masking them.

(9) The bacteria were observed in unstained free-hand sections as also in paraffin sections stained with very dilute carbol-fuchsin and light green. Apart from the region immediately around the wound, they were found only in an intercellular position. They were seen in gall tissue of all ages from the day of inoculation until the gall showed the characters of maturity about 24 days after inoculation.

(10) Isolation and microscopic observations indicated that the bacteria are present in larger numbers than was previously supposed. The treatment of the tissues with mercuric chlorid before plating appears to lower the count so as to give a false impression of the number of bacteria present.

(11) A further control on the location of the crowngall bacteria was made by observing their multiplication in free-hand sections cut under aseptic conditions and embedded in agar, by subsequently isolating them from such preparations, and by establishing their identity with the usual cultural and inoculation methods. Dilution plates poured from such sections have repeatedly yielded thousands of colonies of the crown-gall organism with less than one per cent contaminations.

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PLATE 1

Stems of tomato and tobacco from inoculation experiments, showing the relation of crowngall development to the size of punctures and to the extent of the water-soaked areas surrounding the wounds [1]

A, B, C, D, E.—The relation between the diameter of the inoculating needle and the size of the gall. Photographed 25 days after inoculation. The diameters of the inoculating needles used, expressed in microns, were as follows: On A, 30; B, 57; C, 147; D, 248; and E, 385. Note that the size of the resulting galls increases quite consistently with the size of the needle and the extent of the water-soaked area. ×11/20.

F.—Longitudinal section of a tobacco stem showing the flooding of the intercellular spaces with liquid after a puncture. The water-soaked area (b) appears as a dark region about the puncture (a). ×4/5.

G. Tomato stem showing water-soaked areas resulting from punctures with inoculating needle (outlined with India ink). Photographed promptly after puncturing and making. ×9/10. (The stems G to K, inclusive, show the relation of crowngall development to the water-soaked areas surrounding the wounds.)

H.—A stem treated as G, photographed three weeks after inoculation. The ink lines (retraced in order to appear distinct) show that the margins of the resulting galls coincide approximately with the limits of the original water-soaked area. ×9/10.

I.—A stem that was punctured and, after a half hour, burned in the same place with a red-hot needle. No bacteria were applied. It served, therefore, as a control on J and K. ×9/10. (It appears in I, J, K that galls may develop from water-soaked areas in which the ruptured cells have been destroyed by heat.)

J.—As I, except that the crown-gall bacteria were applied immediately after the punctures were made, that is, a half hour before the burning. Galls appeared in the water soaked regions that were unaffected by the burning. I and J were photographed two weeks after the beginning of the experiment. ×9/10.

K.—As J, but photographed three weeks after inoculation. ×9/10.

[1] These results are interpreted to indicate that the crowngall bacteria are located in the liquid which is released by the wound and which permeates the intercellular spaces about the punctures.

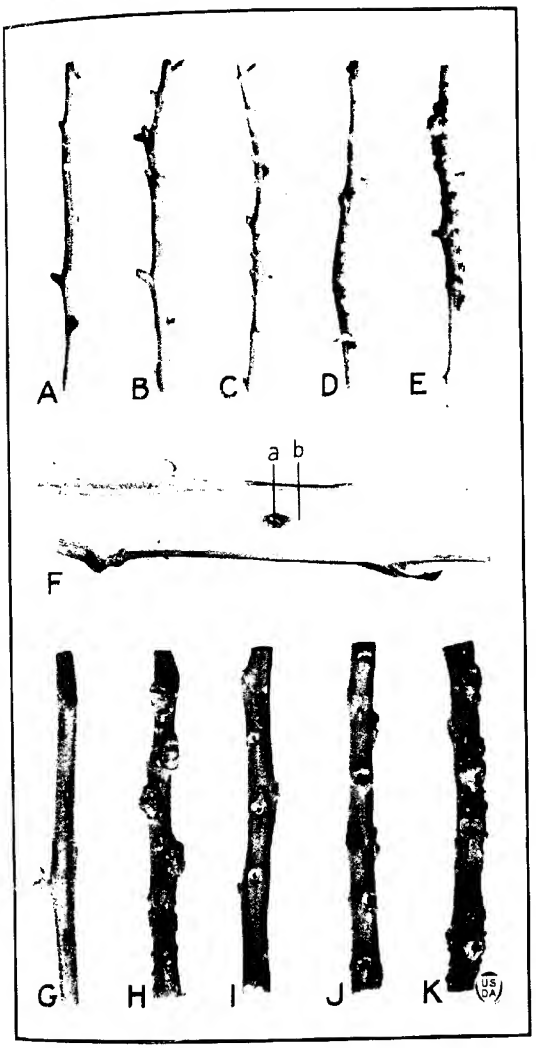
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PLATE 2

Stems from inoculation experiments with reference to the migration of the crowngall organism in the tissues

A to C, inclusive, shows that the bacteria traveled through regions water-soaked by injury and produced galls some distance from their point of entry. In all cases the stems were inoculated only at a.

A, B. The stems were bruised from b to c and inoculated at a. Proliferations developed over the entire length of the injured area. ×2/3 (approximately).

C. The stem was frozen from b to c with carbon dioxid and inoculated at a. Galls developed at intervals. ×2/3 (approximately.)

Figures D to G, inclusive, show that the bacteria may travel through some portion of the vascular system.

D. A section was cut out of the stem and the base of the upper part was submerged in a suspension of the crowngall bacteria. The organisms were recovered by cultural methods at intervals up the stem, which suggests that they passed up through the tracheæ. ×1.5 (approximately).

E. As D except that the stem was frozen and dried with the aim of preventing the passage of the bacteria up the stem except through the dead vessels. The bacteria were recovered in culture from the stem above the frozen region, indicating that they had passed through some part of the vascular tissue. ×1/5 (approximately).

F. A cup, in which a suspension of bacteria was placed, was made from cork, rubber sheeting, and vaseline. An incision was made in the stem under the surface of the suspension and sterile cuts made above and below. ×1/5 (approximately).

G. A stem inoculated as in F. The cup surrounded the stem at d. The sterile cuts which produced galls appear at b. It seems likely that the bacteria which produced the galls at b passed through some part of the vascular tissue. ×2/5 (approximately).

H, I. Stems severely wounded and inoculated by punctures which ruptured some of the vascular bundles at d and a. Sterile punctures were made at intervals above. Galls developed at b which were produced by bacteria which probably passed through some of the vascular elements, and were permitted to escape by the punctures. H, ×2/5 (approximately); I, ×1/2.

PLATE 3

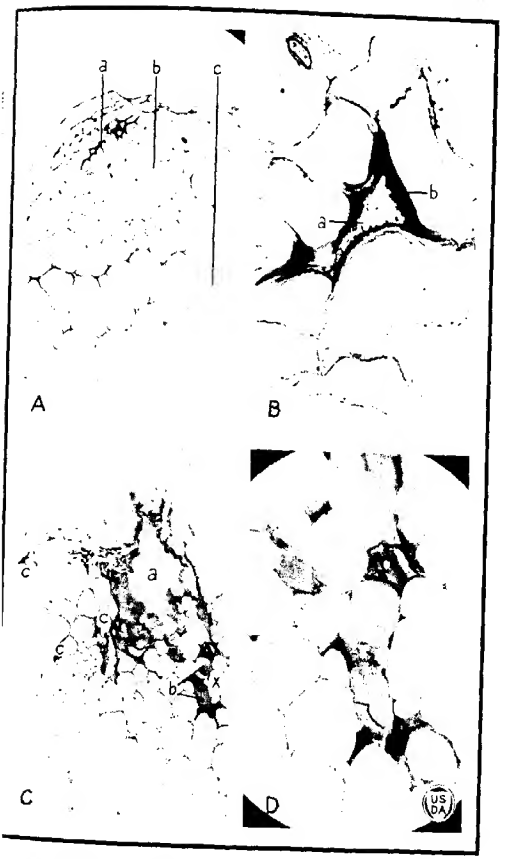
Photomicrographs of crowngall tissue from tomato stem

A. Six days after inoculation. The darkly stained region (a) which occ... the bacteria is surrounded by a more or less circular hyperplastic area (b), and i... bordered by a region of hypertrophy (c). Paraffin section, stained with Flemm... triple stain. ×167.

B.—A darkly stained region on the same slide as section A, but a few sectio... moved, is shown at a higher magnification. In this preparation the bacteria ... masked by the increased intensity of staining in the wall (b) and the substance ... intercellular space. ×600.

C.—Forty-eight hours after inoculation. The large clear space (a) shows wher... needle passed. This is surrounded by masses of bacteria which appear also i... injured cells (b) and intercellular spaces (c). The region at x is enlarged in figu... Paraffin section stained with dilute carbol-fuchsin and light green. ×134.

D.—An enlargement of the region marked x in figure C. Ruptured cells appe... crowded with bacteria that their continued activity seems unlikely. ×400.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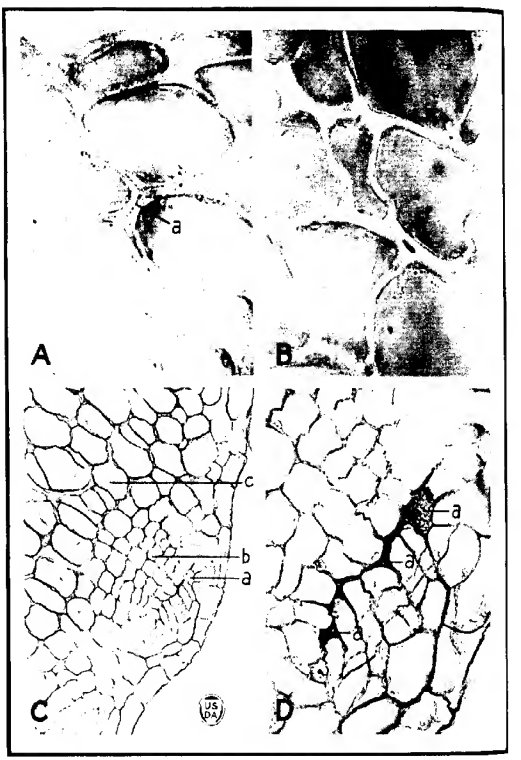
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PLATE 4

Photomicrographs of pectic granules in the middle lamellae and of crowngall bacteria in the intercellular spaces of gall tissue

A. Four-day-old gall. Pectic granules (a) are visible in the middle lamellae between large cortical cells. They have been observed in rapidly growing uninoculated tissue in July. Free-hand section, unstained. ×600.

B. As A, except taken from an uninoculated plant grown in the greenhouse. ×634.

C. Eight-day-old gall. The intercellular spaces (a) are filled with bacteria and surrounded by newly formed regions of hyperplasia (b) and hypertrophy (c). The region containing bacteria is enlarged in figure D. Paraffin section stained with dilute carbol-fuchsin and light green. ×200.

D. An enlargement of the region containing bacteria in figure C. The intercellular spaces which contain the bacteria are maked a. In the slide these walls and the bacteria are stained red while the rest of the tissue shown appears green. ×634.

A and B were photographed in March, 1922.

PLATE 5

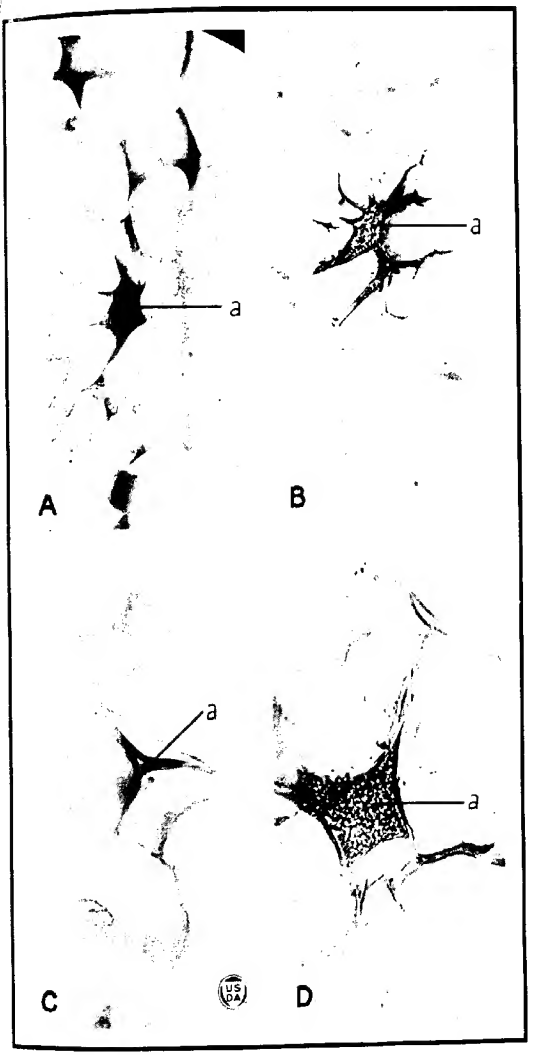
Photomicrographs of the crowngall bacteria in the tissue

A.—Six-day-old gall. The bacteria are seen in the intercellular space mark from which they extend a short distance into the middle lamellae. Paraffin s stained with dilute carbol-fuchsin and light green. ×555.

B. As A, except from a 10 day-old gall. ×617.

C.—As A, except from a 14-day-old gall. The bacteria have acted on the so as to produce a thickening and a change in staining reaction. ×835.

D.—As A, except from a 14-day-old gall. ×917.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YGEN-SUPPLYING POWER OF THE SOIL AS INDI-ATED BY COLOR CHANGES IN ALKALINE PYRO-ALLOL SOLUTION[1]

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INTRODUCTION

It is generally agreed among students of plant physiology and agricul-re that the roots of many plant forms require free oxygen, obtained m the surrounding soil, for their healthy activity. The careful experi-ental work of Free, Cannon, and others, as well as the general experi-ce of agriculturists and horticulturists, indicates that many kinds of ...ts suffer markedly and even die when access of free oxygen to their ...s is cut off. One of the reasons given for cultivating the soil in ...icultural work is that tillage tends to facilitate the entrance of atmos-...ric oxygen into the soil in which the roots are found. It is often ob-...ved that low areas of a field of grain, for example, collect water, which ...ds on the surface of the ground for some time after a rain, and the ...r plant growth frequently noticed in such areas—even after the sur-...e water has disappeared—is often explained by supposing that the ...ling of the soil hindered or prevented the downward movement of ...n from the air.

...m considerations of this kind it is at once suggested that, if health ...o be maintained, the roots of any individual plant must receive ele-...ntary oxygen from the surrounding soil at a rate sufficiently rapid to ...e their physiological processes adequately supplied. As is indicated ...the experiments of Livingston and Free [2] it is to be expected that this ...essary rate of oxygen supply will be found to be different for different ...s of plants and probably for different plants of the same kind grown ...der sufficiently different conditions. It would then follow that a plant ...n otherwise suitable environment would remain healthy only so long ...the conditions in the soil about its roots were such as to allow the ...essary rate of oxygen absorption through the root surfaces. The ...th of the plant would be impaired if the ability of the surrounding ...l to supply oxygen to the roots were sufficiently diminished so that the ...d arrival of oxygen at the root periphery fell below the rate of ab-...ption necessary for healthy activity. It seems clear that a given root ...e can not absorb oxygen any more rapidly than this substance comes ...from the surroundings.

The ability of soil to supply oxygen to an oxygen-absorbing surface, ...as that of a root, might be expected to be greater with relatively dry ...ose soils, and especially near the soil surface, while it would be less ...the more compact or wetter soils and for greater depths. This ...ygen-supplying power of the soil may sometimes be related, in a general ...to the oxygen content of the soil in the vicinity of the absorbing

...r publication May 2, 1923.

2 ...ov, B. E., and FREE, E. E. THE EFFECT OF DEFICIENT SOIL OXYGEN ON THE ROOTS OF HIGHER ... Johns Hopkins Univ. Circ. 293, p. 182–185. 1917.

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surface, but it is surely not generally proportional to the oxygen conc... of the soil in general at the given depth; a soil containing very l... oxygen might still deliver oxygen to the absorbing surface at a considera... maintained rate, while a soil containing much oxygen might soon be... depleted near the absorbing surface so that its maintained rate of deli... would be low. Assuming that roots require an oxygen supply from t... soil, the oxygen condition that determines whether they shall be healt... or unhealthy is therefore not to be defined in terms of the oxygen conc... of the soil at the various depths where the roots occur. The soil feat... that determines the health of the roots, as far as oxygen is concern... must be the oxygen-supplying power. It should be noted that, like o... environmental conditions, this dynamic feature of the plant's subter... nean environment ought to be a limiting condition only when its valu... lower than the necessary rate at which oxygen absorption must proc... if the plant is to remain healthy. As long as the soil is able to sup... oxygen more rapidly than it is required by the roots, it should make... difference—other conditions being adequate for health—how great t... oxygen-supplying power may be.

It seems that ecology and agricultural science would be advanced... we might be able to study and compare the oxygen-supplying power... field soils at various depths, down to the lower limit of penetration... the roots of plants growing in them. The dynamic soil feature to... emphasized has apparently not yet attracted the attention of ecologi... foresters, and students of crop plants; indeed, it seems only to have be... barely mentioned in the literature thus far. It was somewhat surpris... to find no mention of this dynamic consideration in such a thoro... review of the literature of soil aeration as that recently made availa... by Clements's[3] excellent monograph on this subject. In the Year B... of the Carnegie Institution of Washington for 1921 there is a report b... W. A. Cannon,[4] in which he says:

> It is the rate of supply and not the partial pressure of the gas (in the soil air) that is important.

Such studies and comparisons as those just suggested can not, of cours... be begun until some suitable method has been devised for measuring... the oxygen-supplying power of the soil, and the work here reported w... undertaken for the purpose of testing certain suggested methods th... seemed to have some promise in this direction. The results here... reported on the oxygen-supplying power of the soil were obtained dur... ing January and February, 1922.[5]

METHOD.

Any method for measuring the power of the soil to supply oxygen t... an absorbing surface must fulfill two conditions: (1) The absorbin... apparatus must allow oxygen from the surroundings to enter by diffu... sion and not by mass streaming (since there is no mass streaming... oxygen into plant roots), and the absorbing surface must not alte... significantly, as to its ability to absorb oxygen, when the surrounde...

[3] CLEMENTS, Frederic E. AERATION AND AIR-CONTENT, THE RÔLE OF OXYGEN IN ROOT ACTIVITY. ... Washington, D. C. 1921. Bibliography, p. 163-183. (Carnegie Inst. Wash. Pub. 315.)

[4] CANNON, W. A. ROOT-GROWTH IN RELATION TO A DEFICIENCY OF OXYGEN OR AN EXCESS OF CAR... DIOXID IN THE SOIL. *In* Carnegie Inst. Wash. Yearbook 20 (1921), p. 48-51. 1922.

[5] These studies were carried out in the laboratory of plant physiology of Johns Hopkins University, ... the general direction of Dr. M. B. Waite, pathologist in charge, Office of Fruit-Disease Investigat... Bureau of Plant Industry, U. S. Department of Agriculture.

nditions change (otherwise the rate of absorption of oxygen could not taken as a measure of the ability of the soil to supply this element); the oxygen absorbed must be removed and collected in some way that the amount taken in during a test period may be quantitatively etermined.

After some preliminary experimentation a porous-porcelain cylinder osed at one end, such as is used in atmometry,[6] was adopted as a promsing form of absorber. To prevent the streaming of the gas through e porous wall the pores were finally filled with paraffin oil (Nujol). s experience has shown, oil-impregnated porous porcelain seems to be dapted for the taking up of oxygen by diffusion. The cylinder was ound down until the lateral wall, excepting the enlarged rim, was from to 2 mm. thick; the rim was then coated with sealing wax to prevent xygen absorption in that region. The oil treatment was applied by filling the hot cylinder (about 100° C.) and allowing it to stand upright or 12 hours, after which the superfluous oil was removed.

The open end of the cylinder was closed by a rubber stopper holding wo glass tubes, one of which extended through the cylinder nearly to he closed end, while the other terminated just inside the stopper. All joints were sealed with spar varnish, as was also done throughout the remainder of the apparatus which is described in the following paragraphs. Oxygen from the surroundings diffuses through the wall of the absorbing cylinder, tending to make the partial pressure of oxygen in the cylinder cavity equal to the external partial pressure in the immediate neighborhood. De-oxygenated gas (illuminating gas thoroughly scrubbed by alkaline pyrogallol solution, adopted merely because it was convenient and served well for these preliminary experiments, which did not deal with living plants) is passed slowly—60 cc. per hour through the absorber, so that oxygen is removed from the cylinder as rapidly as it diffuses in from the outside. The internal partial pressure of oxygen is therefore always almost nil, while the instrument is in operation. The gas coming from the absorbing cylinder is conducted to a bottle containing the indicator solution, in which the oxygen is all absorbed and measured.

The indicator bottle is of the ordinary wide-mouth form, 4 cm. in diameter and 10 cm. high, closed by a sealed-in rubber stopper, through which pass the glass tubes for the entrance and exit of the gas. The entrance tube reaches to the bottom of the bottle, while the other extends only slightly beyond the stopper on the inside.

Ten cc. of the indicator solution is placed in the indicator bottle. For this solution, which collects the oxygen coming in the gas stream from the absorbing cylinder, and which gives indication of the amount of oxygen thus collected, an alkaline solution of pyrogallol is used. It is prepared as follows: Two stock solutions, one containing 50 gm. of pyrogallol dissolved in 100 cc. of distilled water, and the other having 450 gm. of potassium hydroxid in 500 cc. of distilled water, are mixed with oxygen-free water in the proportions of 1:2:20.

As is well known, an alkaline solution of pyrogallol absorbs elementary oxygen with great avidity, the absorption proceeding with marked rapidity and continuing when the gas is rapidly supplied until large amounts have been absorbed. If made under proper conditions, such a pyrogallol solution is practically colorless before any considerable amount

[6] Livingston, Burton Edward. THE RELATION OF DESERT PLANTS TO SOIL MOISTURE AND TO EVAPORATION. 78 p., 16 fig. Washington, D. C. 1906. Literature cited, p. 77–78. (Carnegie Inst. Wash. Pub. 50.)

of oxygen has been taken up, but with the beginning of oxygen absorption the solution begins to show color, and the color gradually darkens as the process is continued, passing through a series of tints and shades from very pale brownish orange through more intense browns that become redder and finally somewhat purplish as the final color is approached. After much oxygen has been absorbed the solution is nearly opaque and almost black.

A rather prolonged study of the possibilities of using the rate of change in the color of the indicator solution just described, as an indication of oxygen absorption, led to the colorimetric method here used. An arrangement was finally devised by which the indicator solution may be examined and compared with standard color solution from time to time as it gradually darkens during the period of the experiment. One or two standard color solutions, in bottles similar to the indicator bottle, are placed beside the latter for this comparison, and the examination is always made with transmitted light from a 60-watt Mazda incandescent electric lamp. The light reaches the bottles through a diffusing screen of white paper and the bottles and lamp are inclosed so that all light reaching the eye comes directly through the solutions to be compared. As the apparatus is usually operated, three bright windows are visible, each about 5 mm. high and 2 cm. wide, arranged in a horizontal row. The light of the middle window is from the indicator bottle, while that of either of the other two windows is from the corresponding color standard. By this arrangement it is not difficult to determine with great accuracy whether the indicator solution appears darker or lighter than any standard solution with which it is compared, and this determination can be made almost instantly at any time during an experiment.

After many unsuccessful attempts along other lines, Arny's plan[1] of preparing standard color solutions was tried and found to be adequate for the present purpose. Three solutions were prepared, a red one of cobalt chlorid, a yellow one of ferric chlorid, and a blue one of cupric chlorid. By mixing these three solutions in proper proportions and with proper dilutions (as determined empirically) a number of permanently colored mixtures were secured, each one of which represents, with a high degree of accuracy, one of the colors traversed by the pyrogallol indicator solution during the early stages of its oxygen absorption. In the first experiments only two color standards were used. Standard A is very pale, and its color matches that of the indicator solution when a very little oxygen has been absorbed. Standard B is much darker, and was so chosen that its color matches that of the indicator solution at the end of an experiment period of convenient length. The compositions of the two standard solutions thus far used are shown in Table I. A 1 per cent solution of hydrochloric acid is used instead of water.

TABLE 1.—*Composition of two standard color solutions*

Basic compound.	Pale standard solution (A), in 20.5 cc.	Dark standard solution (B) in 20 cc.
	Gm.	*Gm.*
Cobalt chlorid crystals	0.07	1.55
Ferric chlorid crystals	0.54	0.12
Cupric chlorid crystals	0.144	0.55

[1] ARNY, H. V., and RING, C. H. STANDARDIZED COLORED FLUIDS. *In* Jour. Franklin Inst., v. 180, p. 199-213. 1915.

In operation, the outlet tube from the absorbing cylinder is joined by lead tubing to the inlet tube of the indicator bottle, and the outlet of the latter is connected to the intake of an ordinary bottle aspirator. The outlet of the aspirator is joined to the inlet of a series of large scrubber bottles partly filled with alkaline pyrogallol, and the outlet of this series is connected by lead tubing to the inlet tube of the absorbing cylinder. The gas system is thus completely closed. All tubing except-ing the connections to the absorbing cylinder is of glass, with rubber connections.

The aspirator consists of two 8-liter bottles, each closed by a sealed-in rubber stopper bearing two tubes, one of which reaches to the bottom of the bottle, while the other reaches only through the stopper. The two long tubes are joined together by a 2-meter length of rubber tubing. Each of the short aspirator tubes is connected by a Y and a 2-way cock to the closed circulatory system, so that either one of the short aspirator tubes may be operated as inlet to the aspirator while the other is oper-ated as outlet. One bottle stands about 1.5 meter above the other, the upper one having been initially filled with water and the siphon started through the long tube. Water flows out of the upper bottle and enters at the bottom of the lower one. Gas is gradually removed from the lower and transferred through the circulatory system to the upper, the two-way cocks being properly set so that the gas circulates in the direc-tion indicated above. When the upper one is nearly empty the positions of the two bottles are reversed and the two-way cocks are both reversed, so that circulation continues in the right direction. The rate of gas movement through the system is maintained practically uniform by adjusting the relative heights of the two aspirator bottles. A nearly closed cock was introduced into the circulatory system, adjusted to give the required rate of gas movement. The rate of movement was deter-mined from time to time by counting the number of bubbles entering the indicator bottle per minute.

A safety bottle arranged like the indicator bottle was inserted in the system so that the circulating gas traversed it just before entering the absorbing cylinder, and another was inserted in the gas stream imme-diately beyond the indicator bottle. The alkaline pyrogallol solution in these safety bottles did not become colored, thus indicating that the gas entering the cylinder was without oxygen, and that no oxygen from the cylinder escaped being collected in the indicator bottle.

The apparatus was furnished with a somewhat complicated system of tubes, cocks, extra containers, etc., and with a Chapman filter pump to furnish suction when needed in this auxiliary system. By means of this system the two stock solutions—of aqueous pyrogallol and aqueous potassium hydroxid—were prepared and brought together with proper dilution, the indicator bottle and the safety bottles could at any time be emptied, rinsed and refilled with fresh indicator solution, and other necessary operations could be performed, all without the entrance of oxygen into the system at any place excepting via the absorbing cylinder.

The apparatus just described in its essentials was used as follows: The absorbing cylinder is placed in the exposure for which the oxygen-supplying power is to be determined, and the gas stream is started and allowed to continue for several hours, to establish dynamic equilibrium. A fresh charge of indicator solution is placed in the indicator bottle (the bottle is thoroughly rinsed several times with new indicator solution, after the old solution has been withdrawn, the old solution and the rinsing

portions being discharged through the waste conduit), and the col this solution is compared with standard color solution A at freq intervals until the two colors appear alike. When this occurs, the st ing time of the experiment is recorded. The gas circulation contin at the established rate, and the indicator solution becomes gradu darker, until it matches standard color solution B. Then the time experiment ends is recorded. The difference between these two t records gives the number of minutes required for the indicator solut to take up enough oxygen to alter its color from that of standard col to that of B. Since all of this oxygen has come through the walls of absorbing cylinder the time required for this color change may be ta as inversely proportional to the rate of entrance of oxygen into cylinder. It is of course necessary to allow the gas circulation to go for a considerable time—at least 3 to 5 hours—after every change in exposure of the absorbing cylinder, in order to allow the cylinder a the gas spaces of the apparatus to come into dynamic equilibrium w the new surroundings. In practice, readings were generally taken be this equilibrium had become established, and the numerical results in cated the transition from the old to the new conditions of exposure. order to avoid the accumulation of oxygen in the cylinder it has be found best to maintain the gas circulation through the system at times, whether readings are being taken or not.

According to several approximate determinations the color cha just mentioned required about 0.015 cc. of oxygen under the ordin pressure and temperature conditions of the laboratory air. This consta of the apparatus has not yet been precisely determined, however, si its exact value does not enter into the problem dealt with, as will appe below. As far as tests have gone, with the various features of the ap ratus as they were, it appears that small variations in the temperat of the laboratory were without influence upon the results.

EXPERIMENTATION WITH SOIL

In the following paragraphs will be described a series of experime made with a box of garden soil in the laboratory. The results are to b regarded more as an illustration of the way in which the apparatus m be used than as quantitative data on the soil used. They do furni interesting indications as to how the oxygen-supplying power of the s may be expected to differ with different depths, states of packing, a moisture contents. The soil used was a loam, with considerable admi ture of organic matter, and water enough to make it moist but not w such as is commonly used for potting greenhouse plants. The box us was of wood, 30 cm. wide, 40 cm. long, and 30 cm. deep, paraffined o the inside.

The absorbing cylinder with its two lead tubes (1.5 m. long) connect it to the rest of the system, was first placed upright in a glass jar co taining enough mercury to submerge the absorbing portion. The g stream flowed for 48 hours without any alteration in the color of th indicator solution, showing that there were no appreciable leaks in t system. The mercury was then withdrawn from the jar, exposing t external surface of the absorbing cylinder to the air of the room. T rate of oxygen absorption through the walls of the cylinder under thes conditions was rapid enough so that the color change from color standa A to color standard B required 40 minutes. From this it appears th

he cylinder had the power to absorb oxygen from the air at the rate of bout 0.015 cc. in 40 minutes, or about 0.0225 cc. per hour.

The cylinder was next placed horizontally in the soil box on top of a layer f loosely sifted soil 8 cm. deep, after which more soil was loosely sifted nto the box until the layer above the cylinder was 7.5 cm. deep. In placing he cylinder in the soil the lead tubes were bent so that they extended nto the soil to the required depth and then, before reaching the absorb- ng cylinder, extended twice around it in the form of a horizontal ellipse ing about half way between the cylinder and the box wall. This pre- ution was taken so as to avoid possible direct movement of air from he atmosphere to the cylinder along the tube surfaces.

Within three hours of the time the cylinder was placed in the soil its rate delivery of oxygen to the indicator solution showed a noticeable ecrease, and during the next two days this decrease continued. At the nd of that period the time required for the color change was 80 min- tes; that is, the cylinder was absorbing at the rate of about 0.0112 cc. oxygen per hour.

The cylinder was itself able to take up oxygen at the rate of about 0.02 of oxygen per hour—as shown by the air test—and 8 cm. of very osely packed moist soil could supply oxygen to the cylinder only about alf as rapidly as the cylinder could have taken it up if the soil could ave supplied it.

The cylinder was allowed to remain for 40 days in the soil, without sturbance, to allow some settling. The box was covered and no water as added during this period. Twenty-four determinations were then ade during the next 71 hours, with color-change periods of from 68 o 83 minutes, the average being 75.8 minutes. This indicates an oxygen- upplying power of about 0.0119 cc. per hour. Eight cm. of loosely ifted soil was then added to the box, thus bringing the cylinder to a epth of about 16 cm. below the soil surface. A lengthening of the olor-change period was evident after 3 hours, and the period lengthened adually during the next 3 days, as shown by numerous readings taken intervals. During the succeeding 2 days several series of readings ere taken—16 in all—with color change periods of from 84 to 116 inutes, the average being 94 minutes. This indicates an oxygen- upplying power, at this depth, of about 0.0096 cc. per hour.

Water was next added to the soil, corresponding to 6 cm. of rainfall, hich very nearly saturated the soil. This wetting produced shrinkage the soil so that the soil surface came to be only 13.5 cm. above the hulder. A marked increase in the length of the color-change period as shown after 8 hours. Five determinations during the next 18 hours ve periods of from 150 to 179 minutes with an average of 164 minutes, dicating an oxygen-supplying power of about 0.0055 cc. per hour.

The soil was next packed firmly, lowering the surface about 2.5 cm. t without disturbing the tubes or cylinder. A determination made rectly after packing gave a color-change period of 240 minutes, and nother determination immediately thereafter gave a period of 5,050 inutes. The last-named period indicates an oxygen-supplying power only about 0.0002 cc. per hour.

For the particular cylinder used, it appears that, while the oxygen- pplying power at a depth of 8 cm. in loose moist soil was about 0.0119 per hour, the corresponding power at a depth of 13.5 cm. of firmly acked, nearly saturated soil, was only about 1.5 per cent as great. anging the exposure of the absorbing cylinder from a depth of 8 cm.

to a depth of 16 cm., without otherwise altering the soil, changed the oxygen supplying power from 0.0119 to 0.0096 cc. per hour for this cylinder. Wetting the soil diminished this value from 0.0096 to 0.0055 cc. per hour, and packing the wet soil diminished it from 0.0055 to 0.0002 cc. per hour.

The numerical results just given in terms of the lengths of the color change periods and in terms of cubic centimeters per hour for the cylinder used, have been approximately calculated also to terms of cubic centimeters per hour per square meter, cubic millimeters per hour per square meter, and milligrams per hour per square meter. They are shown in all five different ways in Table II. Of course it is understood that these values are all very rough approximations, and they are rounded off to convenient decimals. The calculations have been made by assuming a temperature of 20° C. and a barometric pressure of 76 cm. of mercury. The different lines of the tabulation represent merely different ways of expressing the four different values dealt with.

TABLE II.—*Approximate results of oxygen tests on moist and wet soil*

	Moist, loose soil.		Wet soil.	Packed wet soil.
Depth (in soil) of absorbing cylinder (cm.)	8	16	13.5	[illegible]
Color-change period in minutes	76.0	94.0	164.0	5052.0
Cc. per hour cylinder	0.0119	0.0096	0.0055	0.0002
Cc. per hour square meter	1.5	1.0	0.59	0.02
Cu. mm. per hour per square meter	1300.0	1000.0	590.0	20.0
Mgm. per hour per square meter	1.73	1.33	0.79	0.03

The results of this preliminary study indicate, as was to be expected, that the oxygen-supplying power of the soil for a plant root becomes less (1) as the root lies deeper in the soil, (2) as the moisture content of the soil above the root increases, and (3) as the soil above the root becomes more firmly packed. It may perhaps be estimated that the oxygen-supplying power of the soil about the roots of ordinary agricultural plants may be something like 1 per cent as great when the soil is packed and saturated by heavy rains as it is when the soil has recently been tilled and is not excessively moist.

CONCLUSION

As stated in the introduction, the aim of this preliminary study was to test certain suggested and seemingly promising methods of approach toward the measurement of the oxygen-supplying power of the soil at different depths and under different conditions of soil moisture and of packing. The writers believe that this aim has been attained and that the method described in the foregoing pages may be regarded as at least fairly promising for this kind of measurement. Doubtless many improvements will be made and other methods may be devised, based on the same or different principles and procedures. This paper is to be regarded merely as a report of progress; enough, however, to show very clearly that the dynamic soil feature here considered will not prove to be unusually difficult of measurement whenever ecology, agriculture, and forestry shall have advanced far enough to require quantitative information on the dynamic aspect of soil aeration.

BACTERIAL SPOT OF LIMA BEAN[1]

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INTRODUCTION

During recent years lima bean plants in the vicinity of Madison, Wis., have been affected with a spot disease which was recognized as distinctly different from the blight caused by *Bacterium phaseoli* E. F. S. This disease was first noted in 1917 in several home gardens at Racine, Wis. In August of that year, the disease was also observed to be quite serious in some of the gardens at Madison. Scarcely a plant could be found free from the spotting and some of the plants were affected so badly that most of the blossoms and small pods were shedding. During the autumn of 1917 intensive investigations were begun which were continued during the winter. After this no further observations were made until the summer of 1919. During 1919 the spot appeared on the first leaves, but the plants outgrew it and remained practically free from it throughout the summer. It appeared again in the spring of 1920 and continued to develop throughout the summer of that year but not so seriously as in 1917.

In June, 1921, the senior writer visited several fields of lima beans on Long Island but was unable to find any signs of the spot disease. The plants at this time, however, had only the first two leaves present, and the weather had been dry and hot since the seed was planted. In September of this year both the spot disease and blight were prevalent and, in some cases, serious in the gardens at Madison.

LITERATURE

The literature up to the present time seems to give no description of the disease or of its causal organism, although a bacterial disease of lima beans having at least some characters in common with the spot disease has been mentioned.

The blight attributed to *Bacterium phaseoli* E. F. S. has been known for several years (7)[3] to attack lima bean. Halstead (4), in 1892, reported the occurrence of a bacterial disease of both common beans and lima beans on the farm of a western seed company and stated that the disease had been known on that farm since 1886. Beach (1) described a bacterial disease of lima beans in New York which he suggested as being distinct from the one caused by *B. phaseoli*. Beach's description of the disease in part as follows:

> At first noticed, these spots are never black, but often have a reddish-purple color inclosing an area of light red color. The spots gradually increase in size and develop a straw colored center of dead tissue.

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[2] The writers wish to make grateful acknowledgment to Prof. L. R. Jones, of the University of Wisconsin, for helpful suggestions in the final preparation of the manuscript.

[3] Reference is made by number (italic) to "Literature cited," p. 151.

This description characterizes the disease under consideration in this paper more nearly than it does the blight caused by *B. phaseoli*, as the two diseases have been seen at Madison, Wis. In 1898, Sturgis [5] described a bacterial disease of lima beans in Connecticut which he attributed to *B. phaseoli* E. F. S. However, his description of the disease more nearly characterizes the spot disease than the blight. Manns [5] has also described a bacterial organism which is pathogenic upon certain legumes, including lima beans. He did not describe or illustrate the disease of lima beans, but his report upon cultural and morphological characters precludes the possibility of the two organisms being the same.

SYMPTOMS

Since the bacterial blight caused by *B. phaseoli* also attacks the lima bean, it is well to differentiate between this and the bacterial spot. The spots of the two diseases appear quite different from the beginning and can be easily distinguished even when the two occur on the same leaf or pod as they frequently do.

BACTERIAL BLIGHT

The blight, caused by *B. phaseoli* E. F. S., attacks all the parts of the lima bean plant which are subject to the spot disease. The lesions on all parts of the plant are very similar to those produced on similar parts of the common bean (*Phaseolus vulgaris*) by *B. phaseoli*. Young lesions on the leaves are water-soaked, and the tissues surrounding them soon dry out, causing large blighted areas which generally extend to the leaf margin. Exudate appears on the lower surface of the young lesions and dries out, forming a thin film or scale. The blighted areas are not as brown in color as the bacterial spot (Pl. 1, A), but the small veins near the spots are often red. On the pods and stems the young lesions are also water-soaked and accompanied by films or crusts of yellow exudate. The lesions extend through the walls of the pod and attack the seed. A thick yellow exudate is often produced both outside of and beneath the seed coat.

BACTERIAL SPOT

On the leaves where this disease is very conspicuous, it is characterized by brown to purplish-colored lesions, which vary from somewhat irregular to almost circular shapes (Pl. 1, B). The spots are never at any time water-soaked. At first they appear as small brown points on the upper surface and enlarge very rapidly for the first few days. On the lower surface the young spots are depressed and the margins are a lighter color than on the upper surface. As the spots enlarge, the centers dry out and turn gray or straw colored and the margins remain a glistening, purplish red color. Single spots are usually from 1 to 3 mm. in diameter and may be quite generally scattered over the leaf surface, although they are occasionally grouped and confluent, especially on the first leaves. When spots thus coalesce, lesions several millimeters in diameter are formed. In the latter case the centers of the lesions break out and give the leaves a ragged appearance. The lesions are usually smaller and more regular in shape on the upper leaves. On the King of the Garden variety the spots cause a curling or distortion of the young leaves. The disease also occurs on the veins and petioles where it appears as characteristic reddish-brown or glistening caramel-colored streaks. The streak usually occurs

on the upper or grooved side of the petiole, and may extend throughout its length. When it attacks the base of the leaf stalks, the leaves shed prematurely.

The disease is not confined to the leaves but also occurs in the stems and pods. On the stem the lesions vary in size from 1 mm. to several centimeters long (Pl. 2). The lesions on the stems as well as on the petioles extend into the vascular tissue, but no evidence was found to indicate that the organism progressed very far through the vessels. A straw or light wine-colored exudate appears on the stem lesions in the moist chamber and occasionally in the field, which dries down and forms a thin glistening crust. In several cases the peduncles were found attacked and even completely girdled. In such cases, and when the pedicels are attacked, the blossoms and young pods shed. Brown spots have been observed on the blossoms of diseased plants, but the organism has never been isolated from them.

On the pods the disease begins as small brown spots surrounded by a water-soaked halo. The lesions may occur both on the side of the pod, and also along the sutures where they become streaks (Pl. 3, A). They may extend through the walls of the pod and attack the seed, in which case the veins of the seed coat around the invaded area often exhibit a reddish color and occasionally a white sticky exudate underneath the seed coat. In some cases spongy excrescences were found protruding from the inner walls of the pod beneath the surface lesions. A crust of exudate was found on some of the pod lesions in the field. When diseased pods were kept over night in a moist chamber, drops of a straw-colored exudate appeared on the lesions (Pl. 3, B). These drops later dried down to form a thin crust.

Isolations made from stems, leaves, and pods, such as here described, have produced typical lesions when applied to healthy plants and reisolations have yielded the typical organism.

SEASONAL OCCURRENCE

Since the bacterial spot of lima bean has been under observation a variety of conditions has been found to exist. In 1917 the disease appeared on the first leaves and progressed steadily throughout the season until frost killed the plants. In 1918 no observations were made. Again in 1919 the disease appeared early in the season, but its development was checked in July with the onset of dry weather and the subsequent growth of the plants was free from the disease. On the other hand, the blight caused by *B. phaseoli* was common in lima beans at Madison, especially in the phytopathological garden where the lima beans were planted beside common beans. Practically the same conditions obtained in 1920. In 1921 the disease did not appear until later in the summer, but once started, it continued to develop until frost. By this time the plants were very ragged. It thus appears that frequent rains and favorable temperature are necessary for dissemination of the organism and development of the disease.

ECONOMIC IMPORTANCE

Under field conditions as observed in 1917 bacterial spot is an important disease of the foliage. Practically no pods were set in the phytopathological garden after August 15 because of the severe infec-

tion of leaves and pedicels. When free from disease, the plants continue to grow and set pods until frost, which in the vicinity of Madison, Wis., is usually not earlier than September 10. Such an outbreak in commercial fields would cause a considerable reduction in yield. Aside from this epidemic, however, the disease has been of little practical importance except late in the season of 1921, when it caused heavy infection of the leaves.

Except for the probability of the organism being carried over on the infected seed, nothing is known of the method of overwintering. In Wisconsin, even though the young seedlings become infected, it appears that subsequent weather conditions are, as a rule, unfavorable for further development of the disease. With such sporadic outbreaks the disease will probably never be of any great economic importance under Wisconsin conditions. In States where the lima bean is grown commercially the disease should be considered a factor of economic importance, provided that the climatic conditions are favorable for its development.

THE ORGANISM

ISOLATION

Microscopic observations of sections of young lesions show the invaded tissue to be swarming with bacteria. The organism was isolated from the leaf tissue in practically pure culture by dipping the diseased tissue in 95 per cent alcohol for an instant, immersing in mercuric bichlorid (1 to 1,000) for one minute, rinsing through three or four sterile water blanks, and crushing in a tube of beef broth. After one-half to one hour dilution plates were poured from the tube of macerated tissue.

In the isolations from stems and pods a wet-shining, rapidly-growing, yellowish organism almost invariably appeared on the plates along with the white organism, but several inoculation experiments proved that it was not pathogenic. Pure cultures of the pathogenic organism have been obtained by touching a drop of the exudate on pods with a sterile needle and transferring directly to agar slopes.

During the course of investigations a number of strains of the organism have been isolated each year and successful inoculations made with them. A comparison of cultural characters showed that all were quite similar. The first strain isolated, designated 1a, has been studied most intensively and is presented as the type strain.

MORPHOLOGY

The organism is a short rod with rounded ends, usually occurring singly or in pairs in young cultures. Short chains have been observed in old agar cultures and in beef-peptone broth containing 4 per cent sodium chlorid. When stained from 3-day-old beef-peptone agar cultures with gentian violet or Loeffler's methylene blue, the cells measure from 0.3 to 0.7 μ in diameter and from 0.7 to 2.2 μ in length, averaging 0.5 by 1.5 μ.

Both Caesar-Gill's and Duckwall's modification of the Pitfield flagellum stains have shown the organism to be motile by one to several polar flagella (Pl. 3, C). No endospores or involution forms have been observed. Capsules were not demonstrated by Welch's staining method from potato agar, nutrient agar, or nutrient broth cultures. The organism is gram negative and nonacid fast.

CULTURAL CHARACTERS

Unless otherwise specified, all cultures were incubated in the dark at about 25° C., a temperature very favorable for the organism. Color notations have been made in comparison with Ridgway's Color Standards.[1] In most cases acidity of the medium was determined by both Fuller's scale and hydrogen-ion concentration. Phenolphthalein was used as an indicator in the determinations by the Fuller's scale method. The different agar media were made according to E. F. Smith's formulae (6) except that 1.8 per cent of bacto agar was used.

AGAR POURED PLATES

On + 10 potato agar colonies appeared in about 24 hours, and at the end of 4 days were about 4 to 5 mm. in diameter. They were creamy white, glistening, smooth, circular, entire, umbonate, and opaque. Old cultures become a pale olive-buff color. The surface was usually rugose, but may appear more or less smooth. The consistency was butyrous. Buried colonies were lenticular.

On + 10 nutrient agar colonies appeared in about 48 hours, and after 4 days were 3 to 4 mm. in diameter. They were flat, circular, smooth, glistening, butyrous, opalescent, with faint gyrose to marmorated markings. The margins were undulate. After 3 to 4 days the medium began to turn yellowish-green underneath the colonies, and the color gradually diffused out into the medium. Buried colonies were lenticular.

AGAR STABS

Stabs in + 10 potato agar showed abundant surface growth, rather convex, glistening, and creamy white. After several days the growth may spread over three-fourths to almost the entire surface. Scanty growth developed along the line of stab for a short distance. There was no change in the medium.

Stabs in + 10 nutrient agar developed slightly less abundant growth than on potato agar and was flat, opalescent, and glistening. There was scant growth along line of stab for a short distance. The medium was greened.

AGAR STROKES

On + 10 potato agar the slant stroke cultures made an abundant, flat, chinulate, smooth, glistening, butyrous, opaque growth. The color was creamy white when young, but when 10 days old it was pale olive-buff by reflected light. The medium was unchanged.

On + 10 nutrient agar slants growth was slightly slower and less abundant than on potato agar. It was thinner and fluorescent. The surface was smooth and glistening. It was echinulate, translucent, and butyrous. A slight putrefactive odor was produced. The medium was greened, and after about 10 days, the growth was about the same color as the medium.

BLOOD-SERUM AGAR

Stroke cultures on blood-serum agar gave abundant growth. The medium was browned and slowly liquefied. In small tubes containing about 5 cc. of the medium and kept at about 25° C. liquefaction was complete in 4 weeks.

[1] RIDGWAY, Robert. COLOR STANDARDS AND COLOR NOMENCLATURE. 43 p., 53 col. pl. Washington, D. C. 1912.

STARCH AGAR

There was no evidence of diastatic action on potato starch suspended in beef-peptone agar when tested with iodin.

GELATIN PLATES

Colonies appeared in about 36 hours and grew fairly rapidly. They were circular with entire margin. Liquefaction began shortly after the colonies appeared and in 3 days had produced saucer-shaped depressions 6 to 7 mm. in diameter.

GELATIN STABS

Surface growth was abundant in 3 days. There was no growth in lower part of stab. Liquefaction began in about 3 days and was complete in 60 days in tubes containing 10 cc. of medium. It was crateriform at first, but later became stratiform. In tubes uniformly inoculated and incubated at 20° C. for 7 days, liquefaction advanced 10 mm.; in 10 days, 15 mm.; and in 30 days, 27 mm. The liquefied medium was fluorescent greenish, with heavy surface growth which when agitated, came down in flocculent masses, thus forming a heavy white precipitate.

NUTRIENT BROTH

In +10 beef-peptone broth a thin fringed pellicle formed and dropped as a unit after 3 or 4 days. Uniform clouding began in 12 to 24 hours and became heavy and persistent within 3 to 4 days. A putrefactive odor was produced. Abundant precipitate was formed which was viscid on agitation. The medium turned yellowish green after 2 days and was alkaline to litmus. No hydrogen sulphid or ammonia was produced. Erlich's reagent showed a positive reaction for indol after 4 days.

POTATO CYLINDERS

Growth on steamed potato cylinders was pale yellow in color, flat, spreading, and viscid. After 7 to 10 days the cylinders became a vinaceous buff color.

MILK

In plain milk there was no apparent coagulation, but a slight creamy consistency developed after 3 to 4 days. Peptonization began at the top after 2 days and was complete at the end of 12 to 14 days. A heavy white, granular precipitate developed. After digestion the medium was honey-yellow in color, of thin gelatinous consistency, and strongly alkaline.

METHYLENE BLUE MILK

The blue color was reduced in 2 or 3 days, but a pale olivine color appeared in the cleared, digested part after about 5 or 6 days. After about 1 month the medium became honey-yellow and viscous.

LITMUS MILK

Alkaline reaction appeared before beginning of protein digestion and persisted until the litmus was reduced.

COHN'S SOLUTION

In Cohn's solution there was no growth.

FERMI'S SOLUTION

A thin fimbriate pellicle was formed and sank to form a flocculent precipitate. Heavy uniform clouding occurred within 3 to 4 days. The medium became milky fluorescent within the first 3 or 4 days and later became a bluish green color by reflected light.

TSCHINSKY'S SOLUTION

The action was similar to that in Fermi's solution except the pellicle was more viscid and the bluish color was less prominent.

LITMUS AGAR WITH SUGARS

Dextrose, maltose, and lactose, respectively, were sterilized in concentrated water solution and added to sterilized litmus agar in sufficient quantities to make a 1 per cent sugar solution. Five cc. of a saturated water solution of azolitmin was added to each liter of the neutral nutrient agar.

Litmus lactose and litmus maltose agar slopes produced good growth. A very faint reduction of litmus occurred at the bottom of the slope after 2 weeks, but the normal color soon reappeared and continued exactly like the controls throughout the duration of the experiment, about 3 months.

On litmus-dextrose agar slopes there was abundant growth and distinct acid reaction. In 3 days the medium began to redden underneath the growth at the base of the slope and this coloration progressed for some depth into the medium. Reduction began underneath the heaviest growth and was complete within 2 weeks. The medium was then an amber or reddish brown color. After about 17 days the litmus color completely reappeared and remained about the same color as the controls.

DUNHAM'S SOLUTION

Moderate uniform clouding developed in 3 days. No ring or pellicle developed. A flaky precipitate formed which broke up when agitated.

RELATION TO OXYGEN

The organism is aerobic. No growth developed in agar in Roux tubes. Tubes of nutrient agar were also inoculated while melted, and rolled to distribute the organism throughout. No growth developed at a depth greater than 1 mm. below the surface. No clouding developed in the closed arm of fermentation tubes with any of the sugars tested.

FERMENTATION TUBES

The tests were made in 2 per cent peptone solution (+12 Fuller's scale, P_H. 8.2 to 8.4) with 2 per cent, respectively, of each of the following carbon compounds: glycerine, mannit, dextrose, lactose, maltose, and saccharose.

The sugars and broth were sterilized separately at 12 pounds pressure and the desired quantities mixed in the culture tubes and flasks under aseptic conditions. Eight tubes were used for each sugar. Clouding began after 24 hours. At the end of 6 days heavy clouding was noted in the open end of all tubes and a precipitate later developed. In all cases there was a definite line of demarcation across the inner part of the U

and there was no visible growth in the closed arm. With dextrose the line of demarcation was only slightly above the inner part of the U. No gas formed in the closed arm in any case.

ACID PRODUCTION

In addition to the fermentation tubes used for determining gas production, three 100 cc. Erlenmeyer flasks were prepared in triplicate for each of the sugar solutions. Fifty cc. of the peptone-sugar solution was added under aseptic conditions to each flask. The media were then uniformly inoculated and incubated at 25° C. The acidity was determined by both Fuller's scale and hydrogen-ion concentration methods after different periods of incubation. Twenty cc. of the solution was removed with a sterilized pipette from each flask for the determinations. Of these cultures acid was produced only in those containing dextrose and saccharose. The other cultures became slightly more alkaline as indicated in Table I.

TABLE I.—*Production of acid from sugars and glycerine*

Carbon compounds used in experiment.	Reaction after different periods of incubation					
	Control.		10 days.		30 days.	
	Fuller's scale.	P_H	Fuller's scale.	P_H	Fuller's scale.	P_H
Dextrose	+12	8.0	+14	7.6	+48	4.2
Saccharose	+13	8.2	+22	6.4	+45	4.5
Mannit	+12	8.0	+10	8.2	+16	8.1
Maltose	+13	8.2	.....	.....	+11	8.3
Lactose	+13	8.2	+12	8.4	+10	6.2
Glycerine	+13	8.2	+11	8.6	+11	8.2

NITRATE BROTH

In fermentation tubes nitrate broth gave heavy clouding in the open end and none in the closed end; no gas was formed. A positive test for ammonia was obtained with Nessler's reagent at the end of 2 and 3 weeks. Trommsdorf's reagent gave a negative test for nitrites. Therefore nitrate was reduced completely to ammonia.

DIGESTION OF CASEIN

Clear casein agar plates were inoculated with the type organism. After 3 days a test with 1 per cent hydrochloric acid indicated that the casein was digested around the colonies. After 2 weeks the whole plate was clear, indicating rapid digestion.

VITALITY ON CULTURE MEDIA

Cultures kept in the ice box may be kept alive indefinitely on nutrient agar by transferring every month. The organism may be recovered from 2 to 3 months old cultures on potato or nutrient agar by transferring to nutrient broth. At laboratory temperature potato and nutrient agar cultures lose their viability after 2 or 3 months.

OPTIMUM REACTION AND TOLERATION LIMITS

Beef-peptone bouillon was adjusted to each of the following reactions with sodium hydroxid and hydrochloric acid: +32, +25, +22, +20, +15, +10, +5, +2, 0, −5, −7, −12, and −16. These were uniformly inoculated with 48-hour-old broth cultures and incubated at 24° C. At the end of 1 week there was growth in all tubes between +25 and −7. Heavier clouding developed at +20 than at +10, but the greenish color did not appear in the +20 medium. A heavy precipitate developed at 0, +2, +5, and +10. At −5 there was only moderate clouding, and very slight growth at −7. The optimum temperature for growth is, therefore, +10 to +20 Fuller's scale.

TOLERATION OF SODIUM CHLORID

Beef-peptone bouillon titrating +15 (Fuller's scale) and containing 0.25, 0.5, 1, 1.5, 2, 3, 4, and 5 per cent, respectively, of sodium chlorid was uniformly inoculated from +10, beef-peptone agar. There was heavy clouding in 0.25, 0.5 and 1 per cent after 2 days, and a thin pellicle was formed. The pellicle would come down intact upon agitation. There was only slight clouding and a small quantity of ropy precipitate in 3 and 4 per cent after 2 days. No clouding was apparent in 5 per cent solution. After 8 days the heaviest clouding was manifest in 0.5 and 1 per cent. There was a pellicle and an amorphous precipitate. There was a ropy precipitate and medium to light clouding in all concentrations except 5 per cent. No clouding occurred in 5 per cent solution, but a small amount of ropy precipitate developed.

TEMPERATURE RELATIONS

In +10 nutrient broth the thermal death point lies between 49° and 50° C. This was determined by inoculating 5 cc. portions of the broth in thin-walled test tubes of 13 mm. diameter, by means of 2 loops of a 48-hour-old broth culture and by allowing them to incubate one-half hour before plunging them into water held at the desired temperature. Five inoculated tubes were held for 10 minutes at each temperature in two different tests and were then plunged immediately into cold water until they were thoroughly cooled. After this they were incubated at 25° C. Good growth took place in all tubes heated up to and including 49° C., but none occurred above that.

The relative optimum temperature was determined by incubating 3 inoculated tubes of nutrient broth and potato agar slants at temperatures ranging from 3 to 39° C. at intervals of two or three degrees. This range was repeated, and a third series was run at the higher temperatures 25°, 30°, 31°, 33°, 34°, 37°, 38° C., respectively. Results were uniform in all three series. After 3 days the optimum growth occurred at 28° to 30° C. in broth, and at 26° to 30° C. on agar. As the period of incubation increased, the maximum growth on agar dropped slowly, appearing at 24–26° C. after 18 days. Below 23° C. the clouding in broth was not so strong and the fluorescence was much more marked than at higher temperatures. Growth was very slow at 3° C., but cultures remained viable for some time at that temperature. At 35° C. there was good growth after the first 24 hours but the increase was slow thereafter and the characteristic color did not develop. Very slight growth occurred above 35° C. After being incubated at 37 to 38° C. for 1 week on potato agar, the organism was killed.

DESICCATION

The organism as it occurs in the host tissues seems to be very resistant to drying. Successful isolations were made from diseased leaves which had been kept in the herbarium 2½ years. On potato agar cultures kept in the ice box, where growth is abundant, the organism was viable after 3 months. When beef-peptone bouillon was inoculated with transfers from these cultures, good growth developed. The organism is very readily killed when dried on sterilized cover glasses. Smears were made from 2-day-old broth cultures on sterilized cover glasses and were placed in sterilized Petri dishes. All cells were dead at the end of 24 hours.

TECHNICAL DESCRIPTION

On the basis of the foregoing studies, the organism is characterized briefly as follows:

Bacterium viridifaciens n. sp.[5]

Cylindrical rods rounded at ends, solitary or occasionally in pairs, in short chains in old cultures; individual rods 0.3 to 0.7 by 0.7 to 2.2μ; motile by one to several flagella; aerobic; no spores; no capsules in agar or beef broth cultures.

Superficial colonies on nutrient agar, circular, smooth, glistening, flat, butyrous-opalescent, with faint gyrose to marmorated markings; margin undulate; medium stained a pale lumiere green.

Gelatin moderately liquefied; no acid produced in milk; casein digested without coagulation; litmus reduced in milk; hydrogen-sulphid gas not produced; nitrates reduced; acid produced in media containing dextrose and saccharose; no growth in Cohn's solution; thermal death point between 49° and 50° C; not acid fast; gram-negative.

Group number 211.23221133.

Pathogenic on varieties of *Phaseolus lunatus* Linn, forming lesions on leaves, stems, and pods.

Type locality: Racine, Wis.

Distribution: Eastern and southern Wisconsin.

INOCULATION EXPERIMENTS

The bacterial spot has been reproduced many times with characteristic symptoms under greenhouse and field conditions by spraying water suspensions of the organism on healthy plants. From lesions produced in this way the original type organism has been repeatedly recovered. The disease could always be produced with a newly isolated strain of the organism by spraying a water suspension of the organism upon uninjured leaves, both old and young ones, and placing the plants in a moist chamber from 12 to 24 hours. Wounds are unnecessary for infection. When carried in culture for several months, the organism became less pathogenic.

Several varieties of lima beans were used in the inoculation experiments including Fordhook, King of the Garden, Dreer's Bush, Burpee's Bush, and Henderson's Bush. All varieties were susceptible, but the spots developed to larger size on the Fordhook than on the other varieties.

Besides these varieties of lima beans, Alaska peas and wax beans were inoculated under both greenhouse and field conditions. No infection developed on any of the plants.

In making the inoculations in the greenhouse the following method was usually employed: Lima beans were planted in previously sterilized

[5] According to Migula's classification and Buchanan's revision (2), the combination would be *Pseudomonas viridifaciens* n. sp.

soil, in 8-inch pots and when the first two or more leaves had developed they were inoculated with an atomizer spray of a water suspension of a 5 to 10-day old culture of the organism. The plants were then placed in a warm, damp chamber for 24 hours, after which they were removed to a greenhouse bench where the temperature was about 22° C.

Practically the same method of inoculation was used in the field. Plants free from the disease were selected, inoculated with atomizer spray, and then covered with a glass moist chamber over night or in humid cloudy weather they were left uncovered. In all these experiments the plants were inoculated before the blossoms began to appear.

Both inside and out of doors typical lesions began to develop in from 2 to 7 days after the plants were inoculated.

Control plants were always sprayed with sterile water and incubated under the same conditions as inoculated plants, but in no case did the disease develop in them.

RELATIONS TO HOST TISSUE

Inoculation experiments have shown that wounds are unnecessary for infection. Razor sections of recently infected leaf tissues showed invasion to be in the parenchyma. Young lesions in leaves were fixed in Gilson's solution, imbedded in paraffin, sectioned, and stained with Ziehl's carbol-fuchsin. A study of diseased material prepared in this way showed that the organism enters through the stomata and works its way into the underlying tissue between the cells. The middle lamella was destroyed and the cells were crowded apart by the increasing numbers of bacteria. In the later stages the walls may be torn or collapse and the bacteria invade the cell cavities. The infected areas dry out and become sunken on the lower surface. No vascular invasion was observed.

OVERWINTERING AND CONTROL

Up to this time practically no experimental work has been done in relation to the overwintering of the bacterial spot organism and the methods for controlling the disease. It has been noted, however, that the disease attacks the pod and seed which suggests the possibility of the organism living overwinter with the seed. In 1917, when severe pod infection developed, only a few infected pods matured. These were picked, and the seed was planted the following spring in the greenhouse, but no infection appeared on any of the plants.

It should not be inferred from this one experiment, however, that diseased seed is not a source of primary infection. Since a pathogenic strain of the organism has been isolated from diseased leaves kept for two years in a herbarium, it seems quite possible for the organism to live over winter in the diseased seed as well, and possibly in the diseased leaves in the field. It has been noted also that the disease appeared year after year in the same garden in the first young leaves, but rarely became serious until later in the summer when both old and young leaves were attacked. This condition is not universal, since gardens have been found free from the disease early in the season and several gardens under our observation have remained free from it throughout the entire year.

Until further work on overwintering and dissemination has been done, it seems inadvisable to recommend any specific control measures. Since the causal organism of bacterial spot attacks the seed, seed disinfection

at once suggests itself. However, many of the seed lima beans purchased on the market have cracked seed coats and will not withstand treatment with mercuric bichlorid or formaldehyde solutions of sufficient concentration and duration to kill the bacteria harbored in the tissues. Seeds with unbroken testa will withstand only a mild treatment with water solutions without injury because the testa wrinkles and breaks when wet. Since only a small quantity of seed known to be infected was available, no tests were made to determine an effective method of seed treatment. Observations made in small gardens and inoculation experiments have shown no indication of resistant varieties. These observations, however, have been made on too small a scale to draw any definite conclusions. If the disease should become serious in sections where lima beans are grown on a commercial scale, the development of resistant varieties would seem to be the most feasible means of control.

SUMMARY

(1) The spot of lima bean described in this paper has been observed in the home gardens in the southeastern part of Wisconsin for several years. So far as known, it has not been reported from other sections of the United States.

(2) All varieties of lima beans so far tested are susceptible. No other species of legumes have been infected.

(3) The disease is characterized on the leaves, where it is most conspicuous, by more or less irregular spots with grayish center and definitely delimited purplish borders. In early stages the disease is characterized by purplish or brown spots, slightly depressed on the lower surface. In late stages the center becomes gray. The spots are never water-soaked and no exudate appears.

(4) Petiole, stem, and pod lesions accompany the disease on the leaves. A small amount of exudation has been observed on the pods in the field. When kept in a moist chamber straw-colored exudate appeared on the pods in large droplets.

(5) The disease was severe in its attack on lima beans in the vicinity of Madison, Wis., during the season of 1917. Such attacks occurred under conditions of heavy rainfall and moderately high temperature. During seasons with high temperature and light rainfall the disease was of little importance. While the disease may never be of great economic importance in home gardens, it has the potentialities, under favorable weather conditions, of causing considerable reduction in yield and would necessitate remedial measures where lima beans are grown commercially.

(6) The causal organism is a medium rod motile by one to several flagella which is described as *Bacterium viridifaciens* n. sp. It grows readily on a variety of culture media, producing white, glistening, opaque colonies. Beef extract agar, beef broth, Uschinsyk's and Fermi's solution are turned green. It produces acid without gas in media containing dextrose and saccharose, reduces nitrates, is non-acid fast and gram-negative. It is highly resistant to desiccation in diseased leaves but is killed within 24 hours on sterile cover glasses. There seems to be a gradual loss of pathogenicity when it is grown in artificial culture.

(7) Foliage and stem infection is readily obtained by spraying water suspensions of young cultures on healthy uninjured plants. No pod inoculations have been made.

(8) Since the seed is attacked, it seems very probable that the organism may live over winter on the seed. It is also possible that the organism may live over winter on the bean refuse, as it is known to live more than two years in herbarium material.

(9) Control measures have not been worked out. Because of the organism passing through the seed coat it seems impossible to kill the organism with watery solutions of disinfectants without injuring the seed. In view of this fact, it seems better to rely upon the selection of disease-free seed and development of resistant varieties in localities where lima beans are grown commercially.

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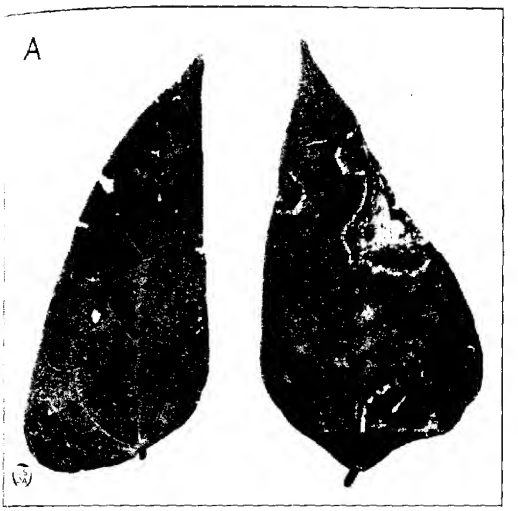
PLATE 1

A.—A comparison of bacterial spot (left) with bacterial blight (right) in King of the Garden lima bean. The bacterial spot is the result of artificial inoculation with water suspension of *Bact. viridifaciens*. The bacterial blight shows natural infection as it appeared in the gardens in the vicinity of Madison, Wis. About natural size.

B.—Bacterial spot in King of the Garden lima bean. A result of artificial inoculation of plants grown in the greenhouse. Photographed two weeks after inoculation. About natural size.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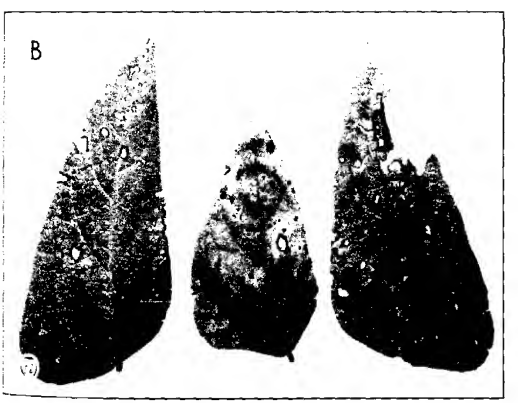
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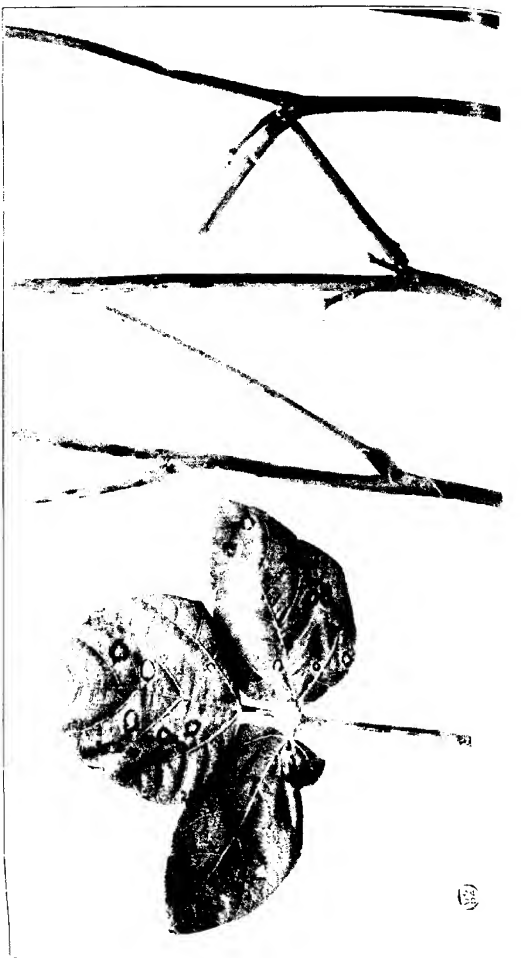


PLATE 2

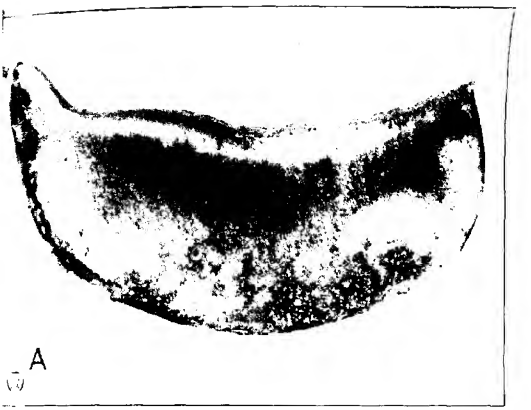
Bacterial spot on leaf and stems of lima bean. Natural infection, showing the characteristic dark borders of the leaf spots and the dark streaks on the stems. About natural size.

PLATE 3

A.—Bacterial spot on pod of lima bean. Natural infection, showing dark lesion extending along the dorsal suture. X 2½.

B.—A portion of lima bean pod affected with bacterial spot. Natural infection showing drops of exudate which came out over night in a moist chamber at room temperature. X 5.

C. Photomicrograph of *Bacterium viridifaciens* from a 24-hour old nutrient agar culture, and stained with the Duckwall modification of the Pitfield method showing flagella. Note that two cells are undergoing the process of division. X 850.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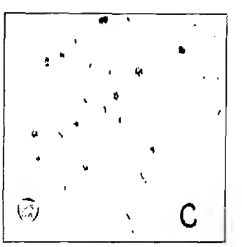
C

HYDROGEN-ION CHANGES INDUCED BY SPECIES OF RHIZOPUS AND BY BOTRYTIS CINEREA[1]

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Investigations with certain species of Rhizopus grown on nutrient solutions have suggested that some of them increase the hydrogen-ion concentration, while other closely related species decrease or have no action on the acidity. These results, obtained more or less incidentally, suggested that no sweeping generalizations could be drawn for all fungi from the results obtained from a few. In fact, they suggested that the results obtained from the study of one or more species could not be applied to all species of a single genus. It has been demonstrated by different investigations that *Sterigmatocystis niger* (*12*),[2] *Aspergillus niger* (*3*), *Penicillium glaucum*, and *Botrytis cinerea* (*9*), *Citromyces pfefferianus*, *C. glaber* (*6*), and others produce acids (not the same acid in all cases) when grown in artificial culture media. Matsumoto (*11*) in a physiological study of 15 different isolations of Rhizoctonia found that the general tendency of these organisms was to increase the actual acidity during growth, the increase seemingly being proportional to the increase in growth.

In investigations carried out by the writers it was shown that when *Rhizopus tritici* Saito was grown on sweet-potato decoction, a vigorous cell-wall-splitting enzym was produced, which separated the cells of sweet-potato disks along the line of the middle lamellae so that coherence was entirely lost. It was found, however, that when a modified Czapek's nutrient solution with glucose as a source of carbon was employed as a substrate, the enzym was not produced but that a certain amount of maceration of the tissue of raw disks resulted, which was found to be caused by the acid formed. The hydrogen-ion concentration was about P_H 1.70 to 1.80. If, on the other hand, pectin obtained from the carrot was substituted for glucose in the substratum, the cell-splitting enzym was secreted and the highest hydrogen-ion concentration was about P_H 3.5. This is some increase in acidity over that of the original solution, but the total acidity was not sufficient to dissolve the middle lamellae.

The writers (*4*) found that the different species of Rhizopus varied considerably in the amount of pectinase produced under identical conditions. They also found that some of the species whose enzym acted readily upon raw disks, decayed sweet potatoes, under natural conditions, quite as vigorously as those which macerated tissue rapidly. With these facts in mind it was suspected that some relationship might exist between the ability of the different species to produce acids and their capacity for decaying sweet potatoes under natural conditions. It was known that *Rhizopus nigricans* Ehrnb. did not decay sweet potatoes as rapidly as *R. tritici;* also that the action of the enzym in the solution from artificial culture on which it grew was very much slower than that of the latter organism. It was thought that this difference between some of

[1] Accepted for publication May 2, 1922.

[2] Reference is made by number (italic) to "Literature cited," p. 163–164.

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the species might be due to the difference in the amount of acid pr duced, which acted alone in the absence of pectinase or as a co-enzy It was accordingly proposed to make a comparison of the changes hydrogen-ion concentration produced by the different species. A stu of the influence of light on some of the vital activities of *R. tritici* was a made.

COMPARISON OF DIFFERENT SPECIES OF RHIZOPUS

A comparison of the different species of Rhizopus growing on swe potato decoction was made. A sufficient quantity of the decoction w prepared at the beginning to carry out the entire investigation. Eve precaution was taken to grow all the species under identical conditio A number of flasks were prepared for each species and several held controls. The inoculated flasks and controls were incubated at a te perature of from 23° to 24° C. in the dark. At the end of the grow period (7 days) the substrate was filtered through cotton to separate from the mycelium. The solution on which each species had grown w collected into one sample and after thoroughly mixing, its hydrogen-i concentration was tested. The control solution (collection from sever inoculated flasks) was found to have a P_H of 5.35.

TABLE I.—*Showing the hydrogen-ion concentration of the solutions on which 11 spec of Rhizopus had grown for 7 days*

Species.	Arto-carpi.	Nigri-cans.	Micros-porus.	Reflex-us.	Tri-tici.	Dele-mar.	Ory-zae.	Nodo-sus.	Arrhi-zus.	May-dis.	Chine-sis.
P_H	4.00	5.44	5.62	3.42	3.07	3.07	3.07	3.07	3.24	3.22	

Table I shows some interesting data. The sweet-potato decoction did not reach as high a hydrogen-ion concentration as is usually obtained in Czapek's nutrient solution. It will be seen that two species, *R. nigricans* and *R. microsporus* van Tieghem, make the solution less acid, while in all other cases the acidity of the solution is increased. A number of the fungi have changed the P_H value to a little above three. Two species, *artocarpi* Racib. and *chinensis* Saito, have brought the P_H of the solution to 4.0 and 4.31, respectively. It is interesting to note the exact similarity in the results obtained with *tritici, delemar* (Boid.) Wehmer and Hanzawa, *oryzae* Went and Pr. Geerlings, and *nodosus* Namysl. These four species are very similar morphologically, and in many cases it is not possible to distinguish between them. The writers have several times called attention to the fact that these four species fall into one group and may eventually be found to be identical taxonomically.

ACIDITY IN RELATION TO MACERATION

In a previous publication (*4*) it was shown that the time required to macerate raw sweet potato disks by the enzym secreted by *Rhizopus nigricans* in the solution on which it grew and that retained by the mycelium, was much longer than that required by such species as *tritici, delemar*, and others. However, *Rhizopus nigricans* is the species responsible for most of the decay of sweet potatoes in storage. It does not, however, decay potatoes as rapidly as some of the other species. Thus far the writers have been unable to offer any explanation of why this species will readily decay sweet potatoes, causing a loss of coherence of the cells in an identical manner, but producing a much less amount of pectinase in artificial cultures. In attempting to offer an explanation of

this interesting phenomenon, data already presented must be briefly reviewed. It has been shown, for example, that *Rhizopus tritici* when grown on a nutrient solution makes the substrate considerably more acid. *Rhizopus nigricans*, on the other hand, renders the substrate less acid. In the case of the former species when growing on sweet potato decoction, the solution is not made acid enough to cause maceration of the cells, so that this can not account for any of the maceration noted.

In order to determine if the acid produced under natural conditions in decaying potatoes bore any relation to the rapidity of decay, a series of experiments were performed in which the rate of maceration by the expressed juice was measured. A number of sweet potatoes were inoculated by the "well method" with *Rhizopus tritici* and *R. nigricans*.

After a definite length of time the juice was pressed from the decayed portion and the hydrogen-ion concentration determined. The time required to macerate raw sweet-potato disks by the enzym suspended in the solution was also obtained. Control flasks in which the juice was boiled to inactivate the enzym were run in every case. The results of these experiments showed that the sweet-potato disks were decayed in considerably shorter time in the juice from potatoes decayed by *R. tritici* than in the juice from those decayed by *R. nigricans*—that is, in 3.5, 4, 2, and 2.5, 4.75, and 5.5 hours respectively, or in an average of 2½ and 4¾ hours.

The hydrogen-ion concentrations of the expressed juice were as follows: *R. tritici*, 4.008 and 4.082; *R. nigricans*, 5.575 and 5.145. The P_H values of the juice of sound sweet potatoes have been found to vary from 5.0 to 6.0 in a considerable number of tests. The results show that *R. tritici* increases considerably the acidity of the cell sap while *R. nigricans* causes little or no change. The juice from the potatoes decayed by *R. tritici* macerated raw sweet potato disks in almost one-half the time required by the juice from those decayed by *R. nigricans*. If, however, enough acid was added to the juice from potatoes decayed by the latter to make it as acid as that from potatoes decayed by the former, the sweet-potato disks were macerated in the two solutions in exactly the same length of time. Previous investigations have shown that the maximum rate of maceration is obtained when the P_H value of the system is between 3.0 and 4.0; that when it reaches 5 or beyond, the action of the enzym is considerably retarded. In other words, the acid in the system appears to function as a co-enzym and serves to hasten the reaction. Judging from these results, it seems that the feeble macerating action of *R. nigricans* when grown in nutrient solutions may be due, in part at least, to its inability to produce acid; also that it would decay sweet potatoes equally as rapidly as *Rhizopus tritici* if it were not for the fact that it fails to provide optimum conditions by the production of an acid.

LIGHT

The studies on the effect of light on the vital activities of *R. tritici* were more or less preliminary to some of the investigations which will be detailed later. The original purpose of this study was to determine if light was in any way connected with the failure of *R. tritici* to produce pectinase in Czapek's nutrient solution, or with the production of acids in this solution in sufficient quantity to cause maceration identical with that caused by the fungus when grown on sweet potato decoction.

The investigations included the influences of light, on (1) the hydrogen-ion concentration of the substrate. (2) the rate of maceration by the

enzym secreted into the solution, (3) the dry weight of fungous material produced, (4) fruiting, and (5) the amount of glucose consumed.

The methods employed in these experiments are briefly as follows. Czapek's modified nutrient solution (*15*) was employed, using 20 per cent dextrose as a source of carbon. Thirty cc. of this solution were placed in each of 210 100-cc. Erlenmeyer flasks, 100 of which, after being inoculated, were held in the dark; 100 were inoculated and held in the light; and 10 were left uninoculated as controls. The flasks were all placed on a laboratory table near a north window. A wooden frame was placed over each set of 100 flasks. The flasks in the dark were covered with three thicknesses of a good grade of satine, and held in place on the top by means of plate glass. Air was admitted at the sides and the light excluded by packing the black cloth closely against the flasks. The top of the frame over the flasks in the light was covered only by the plate glass but the sides were wrapped with white cloth, making them in every way comparable to the sides of the rack covered with black cloth except for the color of the cloth, thus affording the same opportunity for aeration and temperature fluctuations in both sets.

Twenty flasks of each set were removed at the end of 3, 4, 5, 6, and 7 days. The solution from each set was combined into one sample. The sugar present in this sample was determined by means of a Fric saccharimeter. Another portion of the solution was utilized in maceration tests according to methods detailed elsewhere (*4*). The mycelium from each set, after being washed carefully in running water, was collected into one crucible and its dry weight determined. The results from duplicate experiments are shown in Tables II and III.

An examination of Tables II and III reveals no material difference in the results obtained which might be attributed to the influence of light. There was a slight difference in fruiting, but this was not marked. It is interesting to note in this connection that other investigators have found that there is no general agreement among fungi with respect to the influence of light on their growth and fruiting. Lendner (*7*) found considerable disagreement among different members of the Mucorineae and some of the conidial forms of the ascomycetes, while Levin (*8*) showed that total darkness completely suppressed the formation of or greatly reduced the production of pycnidia in some of the Sphaeropsidales. Investigations in still another group of fungi (Agaricaceae) by Maire and de Laroquette (*10*) indicated that here also light has no constant influence on fructification.

The hydrogen-ion concentration of the solutions was considerably increased and the results show that the acid present was sufficient to cause dissolution of the middle lamellae. It was found that after some of the solution on which the fungus had grown was neutralized by the addition of NaOH, it no longer macerated raw disks. There was no difference in the time required to macerate raw sweet-potato disks in the steamed and unsteamed solutions on which the fungus had grown. It will also be seen that there was no maceration in the control solutions, or in the solutions in which the fungus had grown. It seems quite evident from these results that no pectinase was formed, otherwise there would have been some difference in the time required to macerate the disks in the steamed and unsteamed inoculated solutions. What maceration took place in the solutions must be attributed wholly to the acid or to some other substance besides an enzym.

TABLE II.—*Showing the influence of light on the development of Rhizopus tritici*

Time after inoculation.	Light. Ph.	Amount of dextrose.	Rate of maceration by the solution. Not steamed.	Steamed 15 minutes.	Dry weight of mycelium.	Fruiting.	Dark. Ph.	Amount of dextrose.	Rate of maceration by the solution. Not steamed.	Steamed 15 minutes.	Dry weight of mycelium.	Fruiting.	Uninoculated control. Ph.	Amount of dextrose.	Rate of maceration by the solution.
Days.		*Per cent.*			*Gm.*			*Per cent.*			*Gm.*			*Per cent.*	
3	2.18	15.5	Considerable in 48 hours.	Same as not steamed.	0.0327	None	2.08	15.2	Slight, in 48 hours.	Same as not steamed.	0.0418	None	4.23	15.9	None.
4	1.84	14.75	Well advanced in 48 hours.	do	0.0855	do	1.84	14.65	Nearly complete in 48 hours.	do	0.0842	do	4.39	15.9	Do.
5	1.81	14.7	Complete in 24 hours.	Complete in 24 hours.	0.0939	do	1.81	14.45	Complete in 24 hours.	do	0.0956	do	4.38	15.9	Do.
6	1.82	14.6	do	do	0.0944	do	1.80	14.45	do	do	0.105	do	4.40	16.1	Do.
7	1.82	14.8	do	do	0.094	do	1.82	14.7	do	do	0.1014	do	4.21	10.1	Do.

TABLE III.—*Showing the influence of light on the development of Rhizopus tritici*

Time after inoculation.	Light. Ph.	Amount of dextrose.	Rate of maceration by the solution. Not steamed.	Steamed 15 minutes.	Dry weight of mycelium.	Fruiting.	Dark. Ph.	Amount of dextrose.	Rate of maceration by the solution. Not steamed.	Steamed 15 minutes.	Dry weight of mycelium.	Fruiting.	Uninoculated controls. Ph.	Amount of dextrose.	Rate of maceration by the solution.
Days.		*Per cent.*			*Gm.*			*Per cent.*			*Gm.*			*Per cent.*	
3	2.12	15.25	None in 48 hours.	None in 48 hours.	0.0413	None	1.90	14.6	Complete in 24 hours.	Complete in 24 hours.	0.0729	None	4.65	15.5	None in 48 hours.
4	1.86	14.8	Complete in 24 hours.	Complete in 24 hours.	0.0889	Some	1.82	14.3	do	do	0.0975	Some	4.59	15.6	Do.
5	1.82	14.8	do	do	0.0871	Considerable	1.82	14.3	do	do	0.098	Considerable	4.78	15.6	Do.
6	1.82	14.9	do	do	0.0899	Slightly more than in dark.	1.82	14.5	do	do	0.0947	Slightly less than in light.			
7	1.82	14.9	do	do	0.0885	Same as in dark.	1.82	14.7			0.0959	Same as in light.	4.64		None in 48 hours.

The change in the amount of dextrose was not large in any case, being approximately the same in the cultures held in the light and in the dark. The weight of the mycelium was not characteristically different, although a little more on an average was actually produced in the dark than in the light. One outstanding feature of these results to which attention should be directed is the short time required for the fungus to reach its maximum growth. If the dry weight of the mycelium produced in 3 days is compared with that produced at the end of succeeding days, it will be seen that the fungus has practically completed its growth in 4 days. The hydrogen-ion concentration of the substrate increases very little after the fourth day. There is also very little decrease in the sugar consumed after that time, which shows that the organism grows very rapidly at first.

The above results show that *R. tritici* greatly increases the hydrogen-ion concentration when grown on Czapek's nutrient solution and that the acidity reaches its maximum in from 3 to 4 days. Additional investigations which will not be given in detail have shown that the solution still remains acid at the end of 10 days and does not tend to become alkaline, as is the case with solutions on which some other organisms have grown. Since *R. nigricans* is a species of great economic importance, similar experiments were carried out with it, the results of which are shown in Table IV.

TABLE IV.—*Showing the P_H value of the solution of sweet-potato decoction on which R. nigricans had grown for different lengths of time*

	Period of growth (hours).								
	0	7	24	31	48	55	76	96	120
R. nigricans:									
Inoculation solution	5.48	5.48	5.07	5.02	5.20	5.22	5.93	6.46	6.31
Control	5.48	5.48	5.48	5.48	5.45	.....	5.45	.....	5.40
R. tritici:									
Inoculation solution	5.05	5.08	4.44	3.88	3.62	.....	3.70	3.59	.....
Control	5.03	.....	.....	.....	.....	.....	5.07	.....	.....

The solution upon which *Rhizopus nigricans* had grown showed slight increases in acidity at the end of 24 and 31 hours, after which the hydrogen-ion concentration began to decrease, reaching a final concentration in 4 days, considerably less than that of the original solution.

INFLUENCE OF BOTRYTIS CINEREA ON HYDROGEN-ION CONCENTRATION

Botrytis cinerea Pers. has been used in experiments in which it was found to produce a substance capable of dissolving the middle lamellae so that coherence of the cells was completely lost. Smith (*14*), Brown (*2*), de Barry (*1*), Nordhausen (*13*), Kissling (*5*), and others have made a study of the parasitism of this fungus and its mode of action. The results obtained by these different investigators are not entirely in harmony. Smith, for example, claimed that in old mycelium of *Botrytis cinerea* as much as 2 per cent oxalic acid was produced, which he suspected to be responsible for some of the conditions noted. He appears to have discovered a twofold action on lettuce tissue, one which results

in the killing of the cells and another which brings about a softening of the tissues. The first is caused, he claims, by the acid, and the latter by one or more enzyms. Brown, on the other hand, was unable to demonstrate a killing action independent of that caused by the macerating principle and apparently leans to the view that the entire action, killing and macerating, is due to one and the same substance. Smith made no mention of the age of the mycelium which he used. Brown used mycelium only 1 or 2 days old, so that it is not likely that acids in any considerable amount were present in it. On the other hand, it is probable that an acid was produced in Smith's cultures if he used mycelium several days or weeks old. The writers have shown that *Rhizopus tritici* does not produce an appreciable amount of acid until after about 3 days. They are aware, however, that this is no indication of what *Botrytis cinerea* may do.

The writers included a study of *Botrytis cinerea* in their investigations for three principal reasons. First, it has been studied by a number of investigators and shown to produce a substance capable of dissolving this middle lamellae of the cells of a number of different hosts; second, it has been shown by the writers that species of Rhizopus, which are not parasitic on the sweet potato, produce a weak macerating enzym when grown in culture. In other words, it was found that certain organisms which were not normally parasites on the sweet potato would produce an enzym or some substance when grown in culture which would act upon the middle lamellae. Whether or not this was a characteristic of all fungi was not known. Therefore, it was decided to make such a study of another organism which had already been shown to produce a cell-wall dissolving enzym and which was not a true parasite of the sweet potato. Third, it seemed necessary to determine whether an acid was produced which would cause a maceration of the cells, as the writers showed to be the case when *Rhizopus tritici* was grown on Czapek's solution.

Several different media were employed because the results of previous investigations showed that *Rhizopus tritici*, although it formed a pectinase when grown on sweet potato decoction, would not do so when cultivated on Czapek's nutrient solution with glucose as a source of carbon. In order to partially eliminate the influence which the substrata might exert on the production of pectinase by *Botrytis cinerea*, six decoctions of vegetable origin, namely, string bean, prune, Irish potato, carrot, turnip, and sweet potato, three synthetic media, Czapek's, Richard's, and Pfeffer's solutions, and beef bouillon were employed. The organisms were grown in 100 cc. Erlenmeyer flasks on 30 cc. of media. There were a number of flasks of each media. After inoculation the cultures were incubated in the dark at 28° C. for 7 days. At the end of the growth period the contents of all the flasks of one set were collected into one compound sample. The hydrogen-ion concentrations of the uninoculated controls and the solutions on which the fungus had grown were then determined. The mycelium was saved from one set of experiments and its macerating power determined. On some of the solutions the growth was very poor and no hyphae were obtained.

The results as expressed in Table V show that *Botrytis cinerea* increased the hydrogen-ion concentration in some solutions and decreased it in others. String bean and Irish potato decoctions were changed to a point on the alkaline side of neutrality, the action of the enzym being

perhaps thereby retarded. The results of these hydrogen-ion determinations show that the final alkalinity or acidity of a solution depends not alone upon the fungus growing upon it, but in part, at least, upon its composition.

TABLE V.—*Showing the change in hydrogen-ion concentration in the solutions, and rate of maceration by the enzym in the mycelium and in the solution on which it grew.*

Medium.	Experiment 1.		Experiment 2.		Experiment 3.			
	P_H.		P_H.		P_H.		Maceration.	
	Control.	Inoculated.	Control.	Inoculated.	Control.	Inoculated.	Solution.	Hyphæ.
String bean decoction	4.83	8.21	4.82	7.62	4.82	8.26	None in 48 hours	Complete in 24 hours
Prune decoction	(1)	(1)	3.85	3.68	3.85	4.62	Complete in 24 hours	
Irish potato decoction	5.52	8.21	5.52	8.68	5.52	8.17	Slight in 48 hours	Complete in 48 hours
Carrot decoction	3.24	5.73	4.88	5.42	4.88	5.55	Complete in 48 hours	Complete in 48 hours
Turnip decoction	4.82	5.68	4.76	6.23	4.76	7.99	Some in 48 hours	Complete in 24 hours
Sweet-potato decoction	(1)	(1)	4.89	4.61	4.89	4.62	Complete in 48 hours	Complete in 24 hours
Czapek's solution	4.03	2.65	4.05	2.69	4.05	2.52	Complete in 24 hours	Complete in 24 hours
Pfeffer's solution	3.53	2.92	3.41	3.14	3.41	3.05	Complete in 48 hours	
Richard's solution	(1)	(1)	3.27	3.12	3.27	3.17	None in 48 hours	
Beef bouillon	(1)	(1)	8.15	8.13	8.15	8.22	.....do.....	

[1] No growth.

There was no maceration of raw sweet-potato disks in portions of the solutions which had been steamed before testing them, except in Czapek solution, which had a final P_H of 2.52. There was a slight loss of coherence of the cells in this solution in 48 hours, which was probably due to the acid present in the solution. A careful study of the table reveals the fact that in no case, neither in the solution nor in the hyphae, was a vigorous cell-wall dissolving enzym produced. Only in one case, that of the hyphae grown in Irish potato decoction, was maceration completed in less than 24 hours, which shows for the most part that the pectinase capable of acting upon sweet-potato tissue is only feebly produced by *Botrytis cinerea*.

These data can not be used to substantiate or refute the work of Smith, who claims to have demonstrated the production of oxalic acid. Undoubtedly, the age of the culture would exercise some influence upon the amount of acid produced and Smith does not state the length of time he allowed his cultures to grow. The writers were unable to detect the presence of acid in the mycelium of a 7 days' growth. The distilled water in which a quantity of mycelium was soaked for 3 hours at room temperature had a P_H value of 6.8, which is practically neutral.

Apparently *Botrytis cinerea* is very similar in certain physiological characteristics to some of the species of Rhizopus, which, although not normally parasitic on the sweet potato, produce an enzym, perhaps in a small amount, capable of dissolving the middle lamellae of sweet-potato disks. *Botrytis cinerea* does not secrete a substance which will act on sweet-potato tissue to the same degree that it will on some other vegetable tissues, as shown by Brown and others. Is there then a variety of pectinases secreted by different fungi which is more or less specific for the tissue of certain hosts? Since it is impossible to isolate these enzyms

n unqualified answer to this question can not be given. All evidence long this line must be based on the action which the enzyms in solution ave on cellular structures. It is a well-known fact that in the decay of ertain vegetables and fruits by such organisms as Rhizopus and Botrytis he cells lose their coherence, probably as the result of the action of an nzym. It is also well known that some fungi will produce such an ac- ion on some hosts and not on others, which suggests the possibility that he macerating principle produced by one fungus may be quite different rom that produced by another.

SUMMARY

(1) The influence exercised by the growth of 11 different species of thizopus on the acidity of the substrate (Czapek's nutrient solution) as studied. It was found that two species (*Rhizopus nigricans* and *R. microsporus*) make the solution less acid. All the other species make more acid.

(2) The expressed juice from sweet potatoes decayed by *R. tritici* was ound to be more acid than that from potatoes decayed by *R. nigricans*. aw sweet-potato disks suspended in the juice of potatoes decayed by *R. tritici* were macerated in a shorter time than those immersed in juice rom potatoes decayed with *R. nigricans*. If the juice in both cases is rought to the same hydrogen-ion concentration, the disks are macerated n the same length of time.

(3) Light exercised very little influence on the production of pectinase.

(4) Changes in hydrogen-ion concentration of the substrate induced y *Botrytis cinerea* were studied for several different media. The results how that the hydrogen-ion concentration of some substrates is increased, hile that of others is decreased.

(5) *Botrytis cinerea*, although not normally a sweet potato storage-rot rganism, produces a small amount of pectinase capable of dissolving ie middle lamellae of raw sweet-potato disks when grown in artificial ltures.

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